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ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF GENERAL MEDICAL SCIENCES
Fiscal Year 1981

U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service National Institutes of Health

ANNUAL REPORT

FY 1981

NATIONAL INSTITUTE OF GENERAL MEDICAL SCIENCES

January 1982

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This year, as previously, the National Institute of General Medical Sciences (NIGMS), the basic science Institute, funded a larger number of individual investigator-initiated research grants than did any other component of the National Institutes of Health. Stable and continued support of such investigator-initiated research is essential to the well-being of the basic biomedical research enterprise, which is the domain of NIGMS, and the Institute is committed to continue such a pattern. The excitement of the basic research areas supported by this Institute, particularly in the fields of genetics, cellular biology, and molecular biology and pharmacology, is described in the reports of the specific programs. By way of summary, it should be emphasized that the NIGMS supports studies which are at the forefront of modern-day biomedical discoveries and from which comes the fundamental knowledge needed to lead to the prevention, treatment, and cure of those diseases of man, studies of which are within the missions of the categorical institutes.

Research Training

Also of importance is research training, support for which is needed so as to assure the nation of a group of highly creative, skilled, and innovative leaders in biomedical research for future generations. The NIGMS research training program attempts to do this. The Institute accounts for two-thirds of the predoctoral trainees supported by all of the NIH.

Research training at NIGMS, as well as at the whole of NIH, is supported under the authority of the National Research Service Award Act (NRSA) of 1974. With the advent of that legislation, predoctoral research training at the NIGMS has been in the form of interdisciplinary or interdepartmental training grants. Predoctoral training grants are designed to provide broad, fundamental research training in the biomedical sciences. The training faculty members are generally drawn from several departments and are chosen on the basis of their excellence in research and research training. A great deal of variation in composition, emphasis, and objectives exists among the various programs. The NIGMS training guidelines are not designed to discourage these differences, but rather to provide trainees access to course work and thesis opportunities in an array of different research areas.

Postdoctoral support is emphasized less by NIGMS and is provided both through individual fellowships and training grants.

The issuance of a rescission budget by the Administration in mid-1981 led to considerable confusion in the funding of training programs. Processing of all training grant awards and determination of award levels remained uncertain for much of the year. Before this rescission was announced, the NIGMS had paid about one-quarter of the noncompetitive training grant awards in all areas at their previously committed levels. As in the past, these awards included trainee-related expenses and indirect costs, and provided for the full number of trainee positions.

When the rescission budget was submitted, the Institute was required to delete all trainee-related and indirect costs and to reduce the number of trainees by 10 percent. A proposed one-third reduction in training funds for FY 1981 accompanied this directive. This budget rescission caused considerable difficulties

in the payment of the remaining noncompetitive training grant awards. With some awards having been previously paid at full amount and with significantly reduced funds available for the balance of the year, the Institute had to reduce the remaining awards drastically, giving rise to severe inequities. In addition to the elimination of funds for trainee-related and indirect costs, support for about one-third of the trainees was deleted from each award. Such severe cuts raised questions concerning default on prior "commitments" and the long-range impact on training which is essential for future NIH research programs.

All of these problems finally were resolved upon passage of the appropriations legislation, wherein the Congress restored much of the research training support. The NIGMS then restored trainee-related and indirect costs and trainee positions to bring all noncompetitive awards at least up to the FY 1980 levels. However, available funds permitted very few new competitive awards to be made in FY 1981.

Despite this uncertain state of support for research training for many years and the particularly difficult problems in the past year, the Institute has maintained its support of training programs of the highest quality, particularly multidisciplinary predoctoral programs. The commitment of NIGMS is to the quality of such training rather than on simply increasing the number of trainees. In this vein, the Institute is continually evaluating its research training programs, particularly the Medical Scientist (M.D.-Ph.D.) Program and the Minority Access to Research Careers (MARC) Program. This year, the latter has added a new component, a predoctoral individual fellowship, which is specifically designed to provide selected graduates of the Honors Undergraduate Research Training Programs with support during doctoral studies. Four such awards were made as a result of the first review period.

Office of Review Activities

The activities of this office were consolidated during FY 1981, and Dr. Anthony A. Rene was appointed Chief of the Office. The four review committees continue to have a variable workload, and several ad hoc groups have also been used.

The combined review load of the office is shown below:

Category	Number of Applications	Requested Dollars (in millions)
Training Grants (T32)	83	$83.5
Centers	8	13.4
Program Projects	9	20.3
MARC Fellowships	47	(dollars not available)

Staff Changes: The following persons have been added to the staff:

Dr. Anthony Demsey, Executive Secretary, Pharmacological Sciences Review Committee
Dr. Irene Eckstrand, Genetics Program
Dr. Harriet Gordon, Executive Secretary, MARC Review Committee
Dr. Robert Wenthold, Cellular and Molecular Basis of Disease Program
Dr. Warren Jones, Cellular and Molecular Basis of Disease and Pharmacological Sciences Programs

Mr. Howard Chernoff, Personnel Officer
Mr. Robert Willcoxon, Management Analyst
Ms. Ellen Casselberry, Public Information Officer
Ms. Doris Parker, Public Information Specialist

The following staff members have left the Institute:

Ms. Sharon Posey
Dr. Martha Panitch

National Advisory General Medical Sciences Council

The terms of Drs. Carl Gottschalk, Elizabeth Hay, and Robert Terry expired at the end of FY 1981. Dr. Sara Ratner resigned after a two-year period of service because of laboratory responsibilities.

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FY 1981 TOTAL SUPPORT BY ACTIVITY/GRANT TYPE/GRANT NUMBER
AND SHOWING PROGRAM AREAS
(Dollars in Thousands)

	CMBD		GEN		PS		PBME		MARC		TOTAL	
	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS
CENTERS												
P50 TYPES 5 & 7	1	504	4	3,559	5	3,484	6	2,352	--	--	16	9,899
TYPES 2 & 9	--	—	1	558	--	—	4	1,877	--	—	5	2,435
TYPE 1	—	--	—	--	—	--	1	513	—	—	1	513
Total Centers	1	504	5	4,117	5	3,484	11	4,742	--	—	22	12,847
RESEARCH PROJECTS												
P01 TYPES 5 & 7	7	3,087	8	4,379	4	1,398	6	3,019	—	--	25	11,883
TYPES 2 & 9	1	823	1	379	1	288	5	2,423	--	--	8	3,913
TYPE 1	--	--	1	678	--	--	--	--	--	--	1	678
TYPE 3*	(6)	184	(2)	123	—	—	(2)	72	--	—	(10)	380
Subtotal	8	4,094	10	5,559	5	1,686	11	5,514	--	--	34	16,853
P41 TYPES 5 & 7	--	--	--	--	--	—	60	817	--	—	60	817
R01 TYPES 5 & 7	740	57,834	755	68,004	296	22,037	207	16,913	--	--	1,998	164,788
TYPES 2 & 9	139	15,234	141	16,533	37	3,687	38	4,862	--	—	355	40,316
TYPE 1	99	8,364	145	13,834	34	2,577	37	3,791	--	--	315	28,566
TYPE 3*	(26)	569	(22)	634	(1)	39	(2)	16	--	—	(51)	1,258
Subtotal	978	82,001	1,041	99,005	367	28,339	282	25,582	--	--	2,668	234,928
R23 TYPES 5 & 7	--	--	--	--	--	--	15	614	--	--	15	614
TYPE 1	--	--	--	--	--	--	6	289	--	--	6	289
Subtotal	--	--	--	--	--	--	21	903	--	—	21	903
R25 TYPE 1	--	—	--	--	--	--	--	—	1	73	1	73
Total Projects	986	86,096	1,051	104,564	372	30,025	374	32,816	1	73	2,784	253,574
OTHER RESEARCH												
R13 TYPES 5 & 7	--	--	1	30	--	--	--	--	--	--	1	30
TYPE 1	1	4	2	6	--	--	--	--	--	—	3	10
Total Other	1	4	3	36	--	--	--	--	--	--	4	40
TOTAL RESEARCH	988	86,604	1,059	108,717	377	33,509	385	37,558	1	73	2,810	266,460

*Supplements to existing awards.
NOTE: Discrepancies in totals due to rounding.

FY 1981 TOTAL SUPPORT BY ACTIVITY/GRANT TYPE/GRANT NUMBER
AND SHOWING PROGRAM AREAS
(Dollars in Thousands)

	CMBD		GEN		PS		PBME		MARC		TOTAL	
	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS
CAREER AWARDS												
K04 TYPES 5 & 7	7	283	7	273	2	78	8	307	--	--	24	942
TYPE 1	--	--	--	--	1	38	1	41	--	--	2	80
Subtotal	7	283	7	273	3	116	9	348	--	--	26	1,022
K06 TYPES 5 & 7	7	228	7	226	1	35	4	130	--	--	19	619
Total Career Awds	14	511	14	499	4	152	13	479	--	--	45	1,641
INDIVIDUAL FELLOWSHIPS												
F31 TYPE 1	--	--	--	--	--	--	--	--	4	36	4	36
F32 TYPES 5 & 7	62	1,165	127	2,426	11	211	5	100	--	--	205	3,904
TYPES 2 & 9	1	20	2	39	--	--	--	--	--	--	3	60
TYPE 1	41	771	56	1,066	20	363	7	136	--	--	124	2,337
TYPE 3*	(12)	33	(11)	26	--	--	--	--	--	--	(23)	59
	104	1,990	185	3,558	31	576	12	236			332	6,359
F33 TYPE 1	--	--	3	89	1	28	--	--	--	--	4	117
F34 TYPES 5 & 7	--	--	--	--	--	--	--	--	10	225	10	225
TYPE 1	--	--	--	--	--	--	--	--	10	237	10	237
TYPE 3*	--	--	--	--	--	--	--	--	(2)	14	(2)	14
Subtotal	--	--	--	--	--	--	--	--	20	476	20	476
F36 TYPE 1	--	--	--	--	--	--	--	--	2	16	2	16
Total Ind. Fell.	104	1,990	188	3,647	32	603	12	236	26	528	362	7,004

*Supplements to existing awards.
NOTE: Discrepancies in totals due to rounding.

FY 1981 TOTAL SUPPORT BY ACTIVITY/GRANT TYPE/GRANT NUMBER
AND SHOWING PROGRAM AREAS
(Dollars in Thousands)

	CMBD		GEN		PS		PBME		MARC		TOTAL	
	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS
INSTITUTIONAL TRAINING AWARDS												
T32 TYPES 5 & 7	80	22,112	57	7,712	52	5,026	43	4,606	38	3,549	270	43,003
TYPES 2 & 9	5	1,635	2	194	2	155	2	196	--	--	11	2,180
TYPE 1	1	84	1	44	2	159	--	--	5	420	9	708
TYPE 3*	--	--	(1)	4	--	--	(1)	21	(2)	19	(4)	44
Subtotal	86	23,831	60	7,954	56	5,339	45	4,824	43	3,987	290	45,934
T35 TYPES 5 & 7	1	100	--	--	--	--	5	117	--	--	6	217
Total, Institutional Trng	87	23,931	60	7,954	56	5,339	50	4,941	43	3,987	296	46,151
TOTAL GRANTS (Research, Career Awards, Individual Fellowships, Institutional Training Awards)	1,193	113,035	1,321	120,816	469	39,603	460	43,213	70	4,589	3,513	321,257

*Supplements to existing awards.

NOTE: Discrepancies in totals due to rounding.

FY 1981 NATIONAL RESEARCH SERVICE AWARDS TOTAL SUPPORT
BY ACADEMIC LEVEL/ACTIVITY/ANNOUNCED AREA
(Dollars in Thousands)

ANNOUNCED AREA	TYPES 5 & 7 NO.	TYPES 5 & 7 DOLLARS	TYPES 2 & 9 NO.	TYPES 2 & 9 DOLLARS	TYPE 1 NO.	TYPE 1 DOLLARS	TYPE 3 NO.	TYPE 3 DOLLARS	TOTAL NO.	TOTAL DOLLARS
Predoctoral Individual (F31)										
MARC	--	--	--	--	4	36	--	--	4	36
Predoctoral Individual (F34)										
MARC	10	225	--	--	7	147	2	14	19	386
MARC (Paid by Other Insts.)	(3)	(61)	(1)	(14)	(1)	(21)	--	--	(5)	(96)
Postdoctoral Individual (F33)										
GEN	--	--	--	--	3	89	--	--	3	89
PHARM-SCI	--	--	--	--	1	28	--	--	1	28
Total, F33's	--	--	--	--	4	117	--	--	4	117
Postdoctoral Individual (F32, F34, F36)										
PATH	1	23	--	--	3	90	--	--	4	113
CELL-MOL	61	1,143	1	20	41	771	12	33	115	1,967
GEN	127	2,426	2	39	56	1,066	11	26	196	3,558
CLIN PHARM	1	21	--	--	--	--	--	--	1	21
PHARM-SCI	10	190	--	--	22	401	--	--	32	592
SYS-INT	3	59	--	--	2	41	--	--	5	101
TRAUMA	--	--	--	--	1	20	--	--	1	20
ANES	2	41	--	--	1	19	--	--	3	60
BEHAVIORAL SCI	--	--	--	--	1	18	--	--	1	18
MARC	--	--	--	--	2	16	--	--	2	16
MARC (Paid by Other Insts.)	--	--	--	--	(3)	(86)	--	--	(3)	(86)
Total, F32's, F34's, F36's	205	3,904	3	60	129	2,443	23	59	360	6,465

NOTE: Discrepancies in totals due to rounding.

FY 1981 NATIONAL RESEARCH SERVICE AWARDS TOTAL SUPPORT
BY ACADEMIC LEVEL/ACTIVITY/ANNOUNCED AREA
(Dollars in Thousands)

ANNOUNCED AREA	TYPES 5 & 7			TYPES 2 & 9			TYPE 1			TYPE 3			TOTAL		
	NO.	POS.	DOLLARS	NO.	POS.	DOLLARS	NO.	POS.	DOLLARS	NO.	POS.	DOLLARS	NO.	POS.	DOLLARS
Predoctoral Institutional (T32)															
CELL-MOL	51	1032	12,897	--	--	--	1	6	84	--	--	--	52	1038	12,981
GEN	36	501	5,787	--	--	--	1	4	44	1	--	4	38	505	5,835
PHARM-SCI	39	372	4,098	2	14	155	2	11	159	--	--	--	43	397	4,411
SYS-INT	30	313	3,570	2	19	196	--	--	--	--	--	--	32	332	3,766
MED-SCI	20	601	8,368	4	104	1,563	--	--	--	--	--	--	24	705	9,932
MARC	38	300	3,549	--	--	--	5	24	420	2		18	45	324	3,987
Total, Predoc. T32's	214	3119	38,269	8	137	1,914	9	45	708	3	--	22	234	3301	40,913
Predoctoral Institutional (T35)															
SHORT-TERM RESEARCH TRNG	5	60	130	--	--	--	--	--	--	--	--	--	5	60	130
Postdoctoral Institutional (T32)															
PATH	10	52	946	1	5	72	--	--	--	--	--	--	11	57	1,018
GEN	21	95	1,925	2	10	194	--	--	--	--	--	--	23	105	2,118
CLIN PHARM	13	43	928	--	--	--	--	--	--	--	--	--	13	43	928
TRAUMA	8	28	664	--	--	--	--	--	--	1	1	21	9	29	685
ANES	5	16	372	--	--	--	--	--	--	--	--	--	5	16	372
Total, Postdoc. T32's	57	234	4,834	3	15	266	--	--	--	1	1	21	61	250	5,121

NOTE: Discrepancies in totals due to rounding.

FY 1981 PBME CAREER AWARD AND RESEARCH GRANT SUPPORT
BY PROGRAM/ACTIVITY/GRANT TYPE
(Dollars in Thousands)

PROGRAM AREA	ACTIVITY	TYPES 5 & 7		TYPES 2 & 9		TYPE 1		TYPE 3		TOTAL	
		NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS	NO.	DOLLARS
ANES	K04	3	113	--	--	--	--	--	--	3	113
	P01	1	796	--	--	--	--	--	--	1	796
	P50	--	--	1	355	--	--	--	--	1	355
	R01	29	2,026	3	295	6	507	1	6	39	2,834
	R23	7	289	--	--	3	141	--	--	10	430
Total Anes.		40	3,224	4	649	9	648	1	6	54	4,528
BIOENGR	K04	3	117	--	--	--	--	--	--	3	117
	K06	3	97	--	--	--	--	--	--	3	97
	P01	2	1,328	4	1,982	--	--	2	72	8	3,382
	P41	60	817	--	--	--	--	--	--	60	817
	R01	118	9,205	25	3,316	20	1,992	1	10	164	14,523
Total Bioeng.		186	11,563	29	5,298	20	1,992	3	82	238	18,935
RADIOL	K04	--	--	--	--	1	41	--	--	1	41
	P01	1	239	1	441	--	--	--	--	2	680
	P50	--	--	1	505	--	--	--	--	1	505
	R01	19	2,097	1	123	5	775	--	--	25	2,995
Total Radiol.		20	2,336	3	1,069	6	816	--	--	29	4,221
TRAUMA	K04	2	77	--	--	--	--	--	--	2	77
	K06	1	33	--	--	--	--	--	--	1	33
	P01	2	656	--	--	--	--	--	--	2	656
	P50	6	2,352	2	1,018	1	513	--	--	9	3,882
	R01	41	3,585	9	1,129	6	517	--	--	56	5,230
	R23	8	325	--	--	3	148	--	--	11	473
Total Trauma		60	7,029	11	2,147	10	1,177	--	--	81	10,352
Total (PBME)		306	24,152	47	9,163	45	4,634	4	88	402	38,037

NOTE: Discrepancies in totals due to rounding.

In FY 1981, this office became involved in a number of interesting and diversified activities described below:

I. Second Conference on Supportive Therapy in Burn Care

On October 9-10, 1980, the NIGMS sponsored a second conference on supportive therapy in burn care. This was a follow-up to the 1978 consensus development conference, and the discussions were devoted to the same topics which had been addressed at that time. They included fluid resuscitation, infection, metabolism, smoke inhalation, and excision. The goals were: (1) to review the research advances achieved since the first conference and to determine the extent to which these advances have altered, if at all, the management of burn patients; (2) to review the impact of the findings and recommendations of the 1978 conference; (3) to recommend ways to assure adequate dissemination of the conference results and to measure the impact of such dissemination on the medical community; and (4) to review the NIGMS trauma and burn research and research training activities.

Research advances since the 1978 meeting led to differences of opinion by the participants regarding certain treatment regimens. In the area of fluid resuscitation, it was felt that any of a variety of formulae can be successfully used in the early care of extensively burned patients providing the amount of fluid administered is sufficient to maintain appropriate perfusion and viability of vital organs and to minimize latent complications. Although isotonic solution was considered the best type for use in most patients, colloid seemed beneficial after capillary permeability had been restored and hypertonic salt solution was preferred with certain other forms of therapy (e.g., topical silver nitrate). There was general agreement that topical application of antimicrobial agents has an important role in the control of bacterial invasion of the wound. Greater emphasis was placed on early postburn excision and the development of a truly synthetic skin. Nutritional therapy, including the effects of specific amino acids and the quantity of protein administered, was considered to have a significant influence on host defense mechanisms. In contrast to the previously strongly held opinion against the use of steroids when there is smoke inhalation injury, the participants at the second conference suggested that a controlled field study be performed to determine the benefits, if any, of a single dose of steroids.

Numerous areas which need further research were cited. These include the development of methods to determine: time, course, and alterations in capillary permeability; mechanisms responsible for breakdown of the host defense system and methods to restore it; molecular mechanisms associated with the catabolic responses to injury; predictors of early parenchymal injury in smoke inhalation; and technology to reduce blood loss following surgical excision of eschar.

The full proceedings of the second conference will be published as a supplement to the August 1981 edition of the Journal of Trauma, and with that journal's permission the proceedings will also appear in the September 1981 issue of the Journal of Burn Care and Rehabilitation.

II. A Study of the Impact of the 1978 Consensus Development Conference

One of the goals of the NIGMS 1978 Consensus Development Conference on Supportive Therapy in Burn Care was to review the impact of the conference on

the care of severely injured patients. To accomplish this goal, the Institute sponsored a study on the impact of the conference recommendations on the six burn care demonstration project sites supported by the Health Services Administration's (HSA) Division of Emergency Medical Services. These six sites, involving 25 hospitals which serve a population of 25 million people, were established by HSA as models to demonstrate the use of the best burn care technology available and therefore were the most likely settings for a technical consensus conference to have its first impact.

The objectives of the study were to determine the following: (1) the degree to which physicians at the six sites were aware of the conference recommendations; (2) the degree to which the conference recommendations were being followed; and (3) whether the recommendations of the conference made a difference in the practice of burn medicine by those who learned about the results of the conference. The results of this study indicate that, overall, the familiarity of the hospital directors with the conference recommendations was very high (96%). All of the directors who responded felt that the conference was important to their clinical practice, and most of those who indicated that they were engaged in ongoing research activities considered the conference important to their research. Further, all of the directors who had heard of the conference felt that such conferences would be useful in the future.

The impact study was presented to the Advisory Committee to the NIH Office of Medical Applications of Research at its July 1981 meeting.

III. NIGMS Task Force on Technology Transfer and Assessment

The NIGMS Task Force on Technology Transfer and Assessment sponsored two seminars in FY 1981, relating to industry/university cooperative programs. These seminars evolved from concerns expressed by members of the task force regarding delays from the development of new technology to the point of use. Of particular concern was the extent to which grant funds should be used to design or improve devices, procedures, or drugs. What are the responsibilities and opportunities for the Federal government at the research/commercialization interface? The purpose of the seminars was to begin to explore the existence of such activities and the concerns and benefits derived from such a program. At one of these, the task force learned about the National Science Foundation (NSF) program for cooperative research projects between universities and industrial firms. The research, however, focuses on fundamental scientific questions rather than technological design and development. Proposals undergo peer review, and NSF relies heavily on university expertise and previous grant support in the areas of study. The second seminar was presented by an NIH grants associate who previously had been a recipient of such a grant. He felt that the transfer of a technology is difficult. The industrial aspect of the interface is mainly involved in the training of users of instruments. Both seminars revealed the need for more motivation on both sides and more involvement in research endeavors by industry. The task force will continue to explore biomedical areas in which the Institute might assist in the transfer of technology.

IV. Presentations and Meetings

The Thirteenth Meeting of the American Burn Association was held in Washington, D.C., April 2-4, 1981. As has been the experience in previous years, NIGMS grantees presented the majority of the research and clinical papers. Of particular

interest was a plenary session entitled "Early Excision as a Team Effort," at which eight of the ten panelists were recipients of research grant support from the Institute. A highlight of the session was the indication that early excision has been accepted by burn specialists to a much greater degree than it appeared to be in 1978 at the consensus conference. At that time the main proponent of the technique was Dr. John Burke, director of the NIGMS burn research center at the Harvard Medical Center. Since then, his success with the procedure has been very convincing. Many of the panelists were in agreement that excisional therapy should begin as soon as the patient is stabilized. Dr. Burke's data revealed that there was a marked decline in the death rate (from 100% to 36%) in patients who had full-thickness burns covering 70% of the total body surface and who were so treated. The hospital stays of these patients were reduced in length by 50%, the percentage of patients requiring postoperative procedures was reduced from 97% to 35%, and the number of operations were reduced from an average of 3.6 to 0.5.

The Assistant Director for Clinical Research participated in a meeting on smoke inhalation sponsored by the National Bureau of Standards (NBS) in April 1981. The topic of the program was the treatment of human victims who have been exposed to toxic levels of smoke. Of particular importance is the need to establish the effectiveness of hyperbaric oxygen, a study currently supported by the NBS.

Also, the Assistant Director participated in a research forum on May 18-19, 1981, for the proposed White House Conference for Children, Youth, and Parents. Her subject was injuries to children and adolescents.

CELLULAR AND MOLECULAR BASIS OF DISEASE PROGRAM

OBJECTIVE

The Program's objective is to gain the fullest knowledge about human cells to assist in the prevention, treatment, and cure of diseases in man. Since living cells generally share fundamental biological, chemical, and physical processes and properties, the Cellular and Molecular Basis of Disease Program supports research using cell and molecular systems from a wide variety of species, tissues, and preparations. The basis of selection is the scientific merit of research using preparations best suited to solving general principles and problems. In pursuit of this objective, the program supports research ranging from precise physical and mathematical approaches in the study of enzyme catalyzed reactions to investigation of the cellular interactions during embryonic development. Results from this broad range of research are reported in many scientific journals, adding important general principles, techniques, and information which contribute directly and indirectly to our expanded knowledge about normal and abnormal cellular functions. It is this fundamental knowledge that undergirds the study of human function and disease. A number of specific examples of the results of this vital role are presented in this report.

PROGRAM SCOPE

The cell is the fundamental biological unit of all living organisms. It holds the capacity for generating, from simple exogenous sources, all its macromolecules, energy, and complex organization and structure. Cells form selective cell-cell associations, continually displaying highly specific intercommunication and interacting with and adapting to environmental factors. Although they start from a single zygote, cells differentiate into a remarkable range of specialized types. The selective expression of the genome in differentiated cells and numerous factors, both internal and external (including interactions with other cells), bring this about.

This differentiation produces an enormous variety of cells in the tissues of higher organisms. Additionally, many cells have been cultured, often through many generations. These defined cell lines have proven of immense research value and supplement studies on primary cell cultures as well as cells in vivo or in intact tissues. Additionally, microorganisms, both prokaryotic and eukaryotic, have distinct advantages, especially in ease of culture and manipulation. Considering the overwhelming variety of life, it is not surprising that normal human tissues are often not used in basic investigations. Moreover, certain experimental preparations have been studied so extensively over extended periods that they are used because of the wealth of accumulated knowledge of their behavior. However, the research frequently progresses to human tissues to answer questions regarding normal human function and disease.

A major discovery of the past half-century has been the existence of subcellular organelles and the revelation of their variety of structure and function. The advent of sophisticated microscopic and other optical techniques has allowed us to visualize these organelles in great, even molecular, detail. Organelles have been isolated and, in some cases, disassembled and reconstructed in vitro. Much

of the focus of the program has been on the role of individual organelles. The CMBD Program supports research into mitochondria, endoplasmic reticulum, the Golgi apparatus, microtubules, microfilaments, and the plasma membrane.

As an example of the scope of the program's support, consider the support of research on plasma membranes. In this program are significant numbers of research grants on: plasma membrane biogenesis and destruction; interactions between membranes of different cells; cell anatomy; membrane physiology, including transmembrane transport; membrane-localized enzymes; the physical chemistry of lipids; primary and tertiary structures of membrane proteins; membrane protein molecular biology; the physics of lipids and lipid bilayers, including the forces holding bilayers together, bilayer electrical properties, and the physical state of lipids in living tissues.

As can be glimpsed from this brief outline, this program supports a wide range of investigations into cells and their components. It draws on techniques from chemistry, physics, and biochemistry. Its grantees may be in departments of physics, chemistry, immunology, microbiology, physiology, or anatomy, among others. As principally a supporter of investigator initiated research, the CMBD Program is limited only by the vision of high quality investigators of the cell and its component organelles and molecules.

PROGRAM ORGANIZATION

The overall CMBD research program is administered by a Director and Deputy Director, and nine other professional staff members. It is organized into two sections and subprogram areas. The professional staff is:

Charles A. Miller, Ph.D.	Director
Vincent E. Price, M.D.	Deputy Director
Vivian F. Dickson	Special Assistant

Cellular Basis of Disease Section:

Bert I. Shapiro, Ph.D.	Chief
Artrice V. Bader, Ph.D.	Program Administator
W. Sue Badman, Ph.D.	Program Administator

Molecular Basis of Disease Section:

Marvin Cassman, Ph.D.	Chief
David P. Beck, Ph.D.	Program Administrator
John C. Norvell, Ph.D.	Program Administrator
Lee Van Lenten, M.D.	Program Administator
Robert J. Wenthold, Ph.D.	Program Administrator

Research training is administered outside the section organization. Drs. Norvell, Price and Miller are responsible for training.

CELLULAR BASIS OF DISEASE SECTION

The emphasis of the Cellular Basis of Disease Section is research on the cell and its subcellular components, including basic research on all types of

cells (i.e., prokaryotic and eukaryotic microorganisms, cells in tissue or organ culture, isolated cells, such as blood cells, sperm and fertilized and unfertilized ova), as well as research focused on specific cells and their function in excised and intact tissues and organisms. Questions and problems explored and pertaining to specific cell types or components are of a fundamental or general nature applicable to other cell types. This section includes research on all classes of basic cellular or subcellular research not expressly directed to the disease oriented mission of a single categorical Institute, as well as cell biology related to several disease or general pathological states.

A large fraction of the research grants in this program, and an increasing number of new proposals, fall into two major categories: studies on cell membranes and studies on cellular motility and the cytoskeleton. The emphasis of the membrane program is on the plasma membrane of cells, but a significant fraction of the effort is devoted to intracellular membranes. The major areas include active and other transmembrane transport, membrane structure,membrane proteins, and membrane biogenesis. The research on cell motility involves studies of motion in cells, ranging from gross cellular movements such as protoplasmic streaming to the bending of cilia and flagella. Emphasis is on movement of and within non-muscle cells. The larger portion of the work supported here involves studies of cell structures such as microtubules and microfilaments: the proteins associated with motile structures, including microtubular-associated proteins, tubulin, myosin, paramyosin, actin and kinases; and, movement during cell division, including the nature of the mitotic spindle as well as the forces generated during division.

Control of other events during the cell cycle is also supported in this section. Questions addressed include the regulation and synchronization of protein and membrane synthesis, and the development of organelles. The program supports a significant number of projects concerned with the mechanism of cell differentiation. Consistent with the Institute's strong slant to basic research, the work on development does not necessarily involve mammalian cells. It does include investigations into a multiplicity of systems, ranging from slime molds to tissue culture, sea urchin eggs to multicellular bacterial systems.

Many other areas are represented to some extent in the Cell Section of the program. Among these are mechanisms of cell fusion, regulation of leukocyte function, studies of cell adhesion, the role of membrane-bound enzymes, and lipid biochemistry. The latter includes measurements of lipid viscosity, enzymatic reactions in lipid in both its liquid and semisolid phases, and the conditions for micellar and vesicular formation.

MOLECULAR BASIS OF DISEASE SECTION

The Molecular Basis of Disease Section supports basic research on enzymology and regulatory processes in intermediary metabolism; structure and function of the enzyme active site, including the involvement of coenzymes and metal ions; mechanisms of oxidative phosphorylation and electron transport as well as the coupling of these processes to other cellular events such as transmembrane transport; physical and chemical studies on the ordered structure of proteins; and theoretical analyses and modeling of systems of biological importance. Essentially all areas of basic biochemistry and physical chemistry of general biomedical significance are included.

A large fraction of the research grants is concerned with enzyme and protein structure and the relationship to function. The program includes many projects on the nature of the active site. Another significant group includes the analysis of metabolic regulation by covalent modification, for example, cAMP-dependent phosphorylation reactions. Many grantees focus on determining primary, secondary, and tertiary protein structures. These projects employ the use of many state-of-the-art sophisticated techniques, including diffraction analysis and spectroscopic measurements. Among the proteins studied in detail are glycoconjugates and the proteins in the electron-transport chain. In this case the spatial organization of the complex of electron transport components involved in ATP synthesis is a subject of intensive study. The level of complexity being studied by such detailed molecular analysis makes the task formidable.

Although not a large fraction of the Molecular Basis of Disease Program, the theoretical research is a valued part of the section. Some studies of great interest address the question of the forces controlling the folding of proteins and the acquisition of stable folded conformations. Similar studies utilizing advanced methods of quantum chemistry are directed at other specific problems such as antibody-antigen interactions and enzymatic reactions. Research on the structure, molecular dynamics and physical properties of water, electrolytes, and hydrogen bonds is also supported.

Much of the research in the Molecular Section involves extensive use of sophisticated instrumentation. Often the highly quantitative procedures used are at the frontiers of technical capabilities. Techniques utilized are nuclear and electron spin resonance, fluorescence, Mossbauer spectroscopy, circular dichroism, and magnetic circular dichroism, Raman spectroscopy, X-ray, neutron and electron diffraction, and computer analysis, data reduction and modeling. The instrumental capabilities developed on research projects supported in this program often are extended to a wide variety of other areas of biomedicine. The most clinical areas of research and even practice may utilize technologies and instruments developed by basic scientists working on physical and chemical approaches to biomedical problems.

RESEARCH TRAINING

The Cellular and Molecular Basis of Disease Program supports predoctoral and postdoctoral research training in a broad range of fields. Approximately one-half of the total NIGMS training effort is administered by the CMBD Program. In turn, the Institute accounts for two-thirds of the predoctoral trainees supported by all of the NIH.

Postdoctoral support, provided both through individual fellowships and training grants, is essential for the long term mission of the CMBD Program since it fosters the development of a cadre of first-class young scientists in the area of cellular and molecular biology. These scientists will be among the leaders in research supported throughout the NIH over the next several years.

Predoctoral training in the CMBD Program is provided in two areas: the Cellular and Molecular Biology (CMB) area and the Medical Scientist Program (MSTP) area. CMBD training support for postdoctorals is provided through training grants of its Basic Pathobiology area and through Cellular and Molecular Biology individual fellowships.

1. Cellular and Molecular Institutional Predoctoral Program

The Cellular and Molecular Biology training grants are unique in their broad coverage of the biomedical sciences, with faculty and students from many academic departments and/or disciplines, such as biochemistry, biophysics, microbiology, biology, cell biology, molecular biology, genetics, anatomy, pathology, physics, chemistry, physiology, pharmacology, neurobiology and endocrinology.

These grants provide support for high-quality faculty and trainees in these fields in 51 separate university programs across the country, with a total of 1,032 positions. All training grants paid after the rescission budget was announced were significantly reduced, but later restored to FY 1980 levels.

During Fiscal Year 1981 none of the ongoing training programs were up for renewal. Since only one new award was made, together with minimal increases in the second year of others in the Cellular and Molecular Biology Institutional Predoctoral Training Program, the overall size of the program expanded by only 40 positions compared with the previous fiscal year.

2. Medical Scientist Institutional Predoctoral Training Program

The goal of the Medical Scientist Program is to assist medical schools and their universities in the development and operation of programs of combined scientific and medical training for highly motivated students of outstanding research and academic potential. Each institution is encouraged to utilize its unique strengths in developing its program, and each candidate's training over a six to seven year period should draw, as needed, on the full range of scientific and medical opportunities available within the institution. Trainees are awarded the combined MD-Ph.D. degree upon successful completion of their medical-research training.

A majority of the Medical Scientist Training Program awards, as in all other training programs, were reduced as a result of the rescission budget. However, these reductions were restored before the rescission budget was altered. The Institute was directed to give deference to this program due to concern over the decreasing number of young clinical scientists. With the renewal of one award, along with scheduled increases in trainee positions in several ongoing programs, the program grew slightly during the past year.

3. Basic Pathobiology Institutional Postdoctoral Training Program

The goal of this program has been to provide to individuals holding a professional degree training to acquire an indepth knowledge of the principles and methods required for research at the cellular and molecular level in normal and diseased states and to provide first-hand opportunities for post Ph.D.s to gain insight and approaches to fundamental problems of human disease.

This has been the most difficult of all CMBD training programs to bring into full fruition--largely because of the difficulty of attracting post-MDs into research careers. Because of this, many programs have concentrated on post-Ph.D.s, and, as a result, the hoped-for dialogue across the basic/clinical interface has been inadequate. A number of these programs have come up for review and some have not not fared well during review. The number of trainee positions in FY 1981 increased slightly over that for FY 1980 with the award of one additional program. In order

to strengthen their programs, several institutions submitting applications during the past year have sought post MD-Ph.D.s who will require a significant period of postdoctoral research training if they are to remain active in research. This is now being encouraged in combination with their residency training in a clinical specialty as well, where possible.

4. Cellular and Molecular Biology Individual Postdoctoral Fellowship Program

The Cellular and Molecular Biology Postdoctoral Fellowships, under the National Research Service Award Act, are provided to enable individuals holding the Ph.D. or a professional doctoral degree to acquire special advance research training to obtain the necessary cross-field knowledge for a research career in cellular and molecular biology. We continue to receive high-quality applications that encompass the entire spectrum of the program in areas such as membrane structure and function, cell motility, differentiation and growth, enzyme catalysis and regulation, protein structure and function, and related biophysics and chemistry that are essential for an understanding of living systems at the cellular and molecular level.

The reduction of training funds has led to a substantial reduction in new postdoctoral fellowship awards. The number of positions supported by this program has been reduced by about one-third over the past year.

TABLE I

Research and Training Areas Supported by the Cellular and Molecular Basis of Disease Program

	No. of Grants*	Dollars (TC)
Cellular Basis of Disease Section		
Cell Regulation, Differentiation & Growth	93	7,698,000
Cell Organization, Motility & Division	79	6,948,000
Membrane Structure & Function	300	27,320,000
Section Total	472	41,966,000
Molecular Basis of Disease Section		
Enzyme Catalysis & Regulation	226	18,312,000
Glycoconjugates	24	2,241,000
Bioenergetics	78	7,818,000
Structural Studies of Proteins & Related Model Systems	161	13,814,000
Diffraction Analysis of Proteins & Related Model Systems	63	6,136,000
Theoretical Studies of Proteins & Related Model Systems	24	1,876,000
Section Total	576	50,196,000
TOTAL RESEARCH SUPPORT	1,048	92,162,000

RESEARCH TRAINING SUPPORT

Category	No. of Grants*	No. of Trainees	Dollars
Institutional Predoctoral			
Cellular & Molecular Biology	51	1,032	12,881,000
Medical Scientist	24	705	9,932,000
Total Predoctoral	75	1,737	22,813,000
Institutional Postdoctoral			
Basic Pathobiology	11	57	1,018,000
Individual Postdoctoral			
Cellular & Molecular Biology	112	112	1,990,000
Total Postdoctoral		169	3,008,000
TOTAL TRAINING SUPPORT		1,906	25,821,000

*active as of 9/30/81

RESEARCH PERSPECTIVES

Basic research is moving at a rapid pace in the areas funded by the CMBD Program as it is in many other areas, such as those supported by the Genetics Program. Over the past decades there has been a near revolution in the approaches and techniques used in molecular and cell biology. In many respects molecular biology has shown the way by revealing the power of the approach to higher processes through *in vitro* systems, especially using a detailed biochemical and biophysical approach. In addition, molecular biology has provided an intellectual framework for approaching questions in development, control and cell maintenance. It also has demonstrated the value of applying genetic techniques in the attack on a wide range of questions.

Another major reason for the marked advances in the CMBD areas is the significant improvement in instrumentation. There has been wide dissemination of sophisticated new techniques so that many problems are now routinely attacked simultaneously by several techniques involving advanced instrumentation.

Some of the major advances in instrumentation are discussed under the specific highlights below, but others deserve some special mention. As an example, although protein purification methods have been continually improved, it remains difficult to purify many cellular proteins in quantities sufficient to sequence. This is especially true of hard-to-purify membrane proteins or phenotypic variants of more common species. Sequencing is of special interest as a first step to understanding function - such as the configuration of binding or active sites, relationship to other proteins in a complex or lipid in a bilayer, or determination of homologies with related proteins. At the same time preparative separation techniques have improved, so have analytic ones. Sophisticated techniques of electrophoresis in both one and two dimensions have been developed. As a result, a surprising variety of cellular proteins has been revealed. Unfortunately, it is not easy to use two dimensional electrophoresis as a preparative method, especially to isolate significant quantities of a protein.

Several investigators at the California Institute of Technology, notably Drs. Dreyer, Hood, Hunkapiller, and Henwick, have attacked this problem with support from, among others, NIGMS. They have recently developed a new method of automated protein sequencing, using gas phase reagents and a minute sample of protein deposited on a porous glass fiber disc. Although this is a prototype instrument, it has already surpassed previous instruments of the spinning-cup design in sensitivity. The most sensitive instruments of the latter type have also been developed in these labs with NIGMS support. The new sequinator can give useful sequence data on as little as 5 pmol of protein and can determine sequences of more than 30 residues on subnanomole quantities purified by SDS gel electrophoresis. Since this is the first instrument of this class, improvements in design and sensitivity can be reasonably expected in the near future. Moreover, much of the limitation on sensitivity of the new technique is the sensitivity of the detector system, since signal/noise ratio is very greatly improved over previous sequinators. The developers are working on coupling this 'gas-solid-liquid' sequinator with a mass spectrometer to allow sequencing of samples below the pmol range, perhaps in the fmol range.

While many scientists have determined protein sequences directly, by serial degradation, another technology has been developing simultaneously. Several

scientists, many supported by NIGMS, have developed methods for DNA sequencing. Thus, if a known messenger RNA for a given protein is isolated, the complementary DNA can be made. This can then be cloned to produce sufficient protein to sequence. With some added analysis, the message can be read and thus the protein sequenced indirectly. Synthesis of protein starting from the cloned DNA can confirm the identity of the message and cDNA. This apparently roundabout method can actually be faster for protein species where sufficient message can be isolated. The new direct protein sequencing technology opens up the possibility of a new hybrid procedure. A minor protein species can be isolated from acrilamide gels and eluted in sufficient quantities by the present 'gas-liquid-solid' instrument to obtain at least a partial sequence. A message or its complement corresponding to this peptide fragment can be synthesized. It in turn can be used as a probe to isolate the complete, authentic message. This message can be used to make cDNA and then sequenced. Such a strategy has already been employed successfully. Not only can very scarce proteins be sequenced, but their DNA can be cloned prior to synthesis of major quantities of the peptide. Although we are just at the start of this technology, the promise is enormous.

Many instrument developments may occur gradually, but the consequences may be a seemingly sudden opening up of new vistas. For example, nucleic acid sequencing was possible before Maxam, Gilbert and Sanger made it nearly routine. Yet the result of a change form "do-able" to routine is immense. In a similar fashion nuclear magnetic resonance techniques have continuously advanced. Ever more powerful fields have been achieved, and more and more probes have become available. Partial orientation of molecules containing certain groups in an NMR field can potentially give information on molecular stereochemistry, internuclear distances, and electronic structure. The most powerful instruments now available can just barely produce detectable effects. However, since the orientation increases as the square of the field, a more powerful instrument may open up an entirely new area. Some investigators have already been able to detect resonances from single amino acids in membrane proteins using such a very high field instrument. Prior to this technique such resolution of membrane proteins could only be obtained in membranes with highly ordered systems, using diffraction techniques such as neutron, X-ray, or electron diffraction. In contrast, the high-field NMR approaches promise to allow observation of large numbers of individual resolved sites in membrane proteins. Other workers have been able to resolve resonances and determine the conformation of the fatty acids throughout lipid bilayers. The conformation of other proteins in solution, including antibiotics, somatostatin, antibodies, and serine proteases, has been determined with far greater resolution using such a high-field machine.

Two better known technological advances, recombinant DNA and hybridoma technologies, will also open up new vistas and pave the way for major advances in the CMBD program. We mentioned above the use of recombinant DNA technology to isolate and clone DNA segments in order to sequence the message and the corresponding protein. It also allows us to study protein synthesis and post-translational modification in vitro, using large quantities of specific message. Additionally, the introduction of known plasmid vectors to bacteria is proving a major tool in studying the role of the protein coded for by that vector as well as the control of the synthesis, modification and degradation of that protein.

The signal characteristic of monoclonal antibody or hybridoma technology is the ability to raise highly specific antibodies--antibodies which will not cross-

react to any protein lacking the small and very defined determinant. This specificity permits the use of the antibodies as labels for histochemical localization or as affinity reagents for purification. In fact, it appears feasible to multiply label larger proteins with several monoclonal antibodies from different clones. This allows the tagging of molecular regions, a technique opening up many exciting possibilities.

The research highlights provided later in the report detail some research areas showing exciting advances within the recent past. Many other areas are emerging or continuing strongly and a few of these merit mention. One area at the border between the Genetics and CMBD programs is post-translational modification of proteins. Several of the highlights deal with this topic: Reed and Kuehn have studied the control of enzymatic activity by phosphorylation. Kreibich and Sabatini have investigated the role of membrane proteases in clipping the terminal sequence from nascent chains. Rothman and Schekman have tangentially addressed the question of glycosylation of intrinsic membrane or secretory proteins. These are examples of two major and increasing areas in CMBD. The first is control and regulation of protein or enzymatic activity through covalent modification, especially phosphorylation. The second is permanent modification, during or soon after synthesis, to produce the mature protein. Some, but not all, of the latter modifications are cotranslational. The nature and stage of glycosylation are active areas of research. Many membrane proteins are glycosylated, but it is impossible to understand their tertiary structure, orientation, membrane insertion, antigenicity, and role without knowing more about this modification.

The work of Dr. B. Trumpower, highlighted below, is particularly noteworthy as an example of the spectacular advancement and branching out of a new facet of an extensively investigated field. As we reported in the CMBD Annual Report for FY 1980, "one of the striking developments in biology in the last decade has been the identification of a totally new class of biological materials, the iron-sulfur (Fe-S) proteins. What is particularly significant is that at least some of these proteins appear to be major constituents of what was thought to be among the best-understood biological systems, the electrontransport chain. The development of this field also demonstrates how sophisticated physical techniques, applied originally to non-mammalian systems, generated results of great importance to the understanding of fundamantal biological processes." The highlight proceeded to cite nine investigators, from nine grantee institutions, for their long-standing contribution. The development of a selective inhibitor, an approach of great promise, is from yet another lab.

Another recent and exciting study of an enzyme in the biological oxidation-electron transfer pathway has been carried out by Drs. B. Chance, L. Powers and others, using the synchrotron at Stanford University. Although it has been known that cytochrome c oxidase (cyt a_3) is the last step in the oxidation-electron transfer pathway, the physical-chemical mechanism of this unique process has remained little known. It also had been known that cyt a_3 contains both copper and iron at the redox center but, again, the details were uncertain. Using the synchrotron, Chance and collaborators have performed extended X-ray fine structure (EXAFS) measurement. They revealed the iron and copper to be in close proximity and linked by a sulfur bridge. The information from the EXAFS has been crucial in formulating a model for the mechanism of electron transfer of cyt a_3. The existence of a sulfur bridge is surprising and again underlines the importance of this molecular bond in electron transfer. Finally, now that the EXAF studies

have proven worthwhile for at least some problems, we are on the road to adding an important new tool to the arsenal of molecular analysis.

Another of the highlights below discusses the work of Lester Reed on the control of the pyruvate dehydrogenase multienzyme complex and describes the elucidation of the regulation of the association of several enzymes. The investigators found that the association of the phosphatase with dihydrolipoxl transacetylase is regulated by calcium. This is just one of an increasingly impressive number of cellular processes controlled by the level of intracellular calcium. Calcium can have such an important biological role because intracellular free calcium is very low (10^{-6}M) while extracellular calcium and total (free, bound and sequestered) intracellular calcium is much higher. A small increase in plasma membrane calcium permeability, or the release of calcium from intracellular stores or binding sites can rapidly increase the concentration of free calcium up to a thousand fold. The control of intracellular calcium levels is a vigorous and thriving area of research supported by CMBD. Investigators are studying both active and passive calcium transport through the plasma membrane. Some are synthesizing and analyzing the molecular action of calcium ionophores. Other workers are probing the enzymes, lectins, and organelles which are involved in calcium binding or sequestration. Note, for example, the long-standing research efforts of Dr. Lehninger, cited in one of the highlights.

An entire other cohort of scientists in the program addresses the question of the mechanisms by which calcium regulates cellular processes. In some cases, as in the example of the pyruvate dehydrogenase multienzyme complex, calcium appears to modulate activity directly. Other examples of this category include enzymes for which calcium is a substrate or cofactor, such as phospholipase A_2; in yet other cases calcium may bind to negatively charged lipids in the membrane and work by modifying the electrostatic field. Particularly exciting are the instances in which calcium first acts on a regulatory protein, calmodulin, which in turn binds to other proteins or complexes, affecting their activity in the process. Calmodulin has been demonstrated in a wide variety of organisms. One NIGMS grantee, Dr. B. Satir, has even shown the antigenic identity between mammalian and ciliate protozoa calmodulin. Among the very many enzymes it has been shown to affect are erythrocyte Ca-ATPase, myosin light chain kinase and plant NAD kinase. Calmodulin shows a high level of homology with the muscle filament protein, troponin C, which confers calcium sensitivity on the actin-myosin interaction. From direct comparison with such homologous proteins, the calcium binding sites of calmolulin have been predicted. Metal binding studies have demonstrated that calmodulin has four equivalent calcium binding sites. Binding produces marked conformational changes in calmodulin. Some NIGMS grantees have been elucidating many features of this small, 16,700 dalton, protein. Among these has been the high-resolution crystal structure. Its conformation is similar to that of several other calcium binding proteins, including troponin C. They all seem to have a characteristic shape, or EF-hand, with similar steric placement of binding sites.

It is interesting that troponin C plays a unique and essential role in striated muscle contraction while calmodulin or related proteins may play a central role in controlling some types of cell movement. The CMBD Program has a large number of grants (see table) focusing on cell motility and the related problem of cytoskeletal organization. There is increasing evidence that fluctuations of intracellular calcium activity may regulate these processes, including the

formation of actin and other cytoskeletal filaments as well as ciliary beat. Calmodulin and other calcium sensitive proteins have been implicated in this control.

The study of calmodulin, a protein so important in regulation, is being pursued by grantees of several institutes. The NIGMS, because of its unique role as a basic research support institute, and one with programs in molecular and cellular structure and function, has funded an increasing number of projects concerned with calmodulin and its action. Other cell activities are also controlled by calcium levels and the CMBD program anticipates a substantial increase in the number of grants in this significant, exciting and expanding area.

In order to understand multienzyme complexes, it has proven necessary to develop first the tools and conceptual framework to analyze the structure and function of individual isolated and purified enzymes. As we have progressed, and progress has been extraordinary over the past couple of decades, we are now able to put the units back together. There will be a major expansion in the efforts in this direction in the near future. Reconstitution is advancing and many techniques are now available to insert membrane proteins into model or reconstituted membranes, for example, in order to mimic the action in a living cell more closely. While Dr. Eisenberg, at UCLA, has explored the tertiary structure of melittin (see highlight below), Drs. M. & D. Tosteson, at Harvard, have been able to incorporate it into membranes and show the nature of the channel formed. They have also been able to infer something about its structure in the membrane, namely that tetramers are required to produce channels. Far more complex reconstitutions are now being attempted in many other labs. Just as important, some of the synthesis occurring is intellectual. As we have succeeded in understanding many cellular chemical events, and the molecules responsible for them, we have begun asking questions concerning interactions of these molecules. Expansion in the future will come from both analytic and synthetic directions.

RESEARCH HIGHLIGHTS

Presented below are examples of recent accomplishments from a variety of cell and molecular systems being investigated by grantees of the CMBD Program. These highlights reflect the broad range and kinds of activities currently being supported.

1. Solvent Effects on Biomolecular Structure and Process
GM 24914-07 (David L. Beveridge), Hunter College, CUNY

Dr. Beveridge and his coworkers are studying the structure of liquid water and aqueous solutions of biological molecules. This work employs statistical thermodynamical liquid state computer simulations applied to fairly simple dissolved molecules. These simulations are based on potential functions representative of quantum mechanical calculations of the intermolecular interactions. Although highly theoretical in nature, this research should help explain the unusual properties of aqueous solutions and water structure effects on biomolecules.

Dr. Beveridge has recently reported progress on liquid water structure and on the aqueous hydration of hydrophobic, ionic, and hydrophilic molecular solutes. The structure of liquid water emergent from Monte Carlo computer simulation on

[H_2O] liquid at 25°C was found on the average to be best described as a network of molecules interacting via bent hydrogen bonds, and thus provides independent evidence for two earlier theories--a continuum model and a random network model. In hydrophobic hydration, quasi-clathrate contributions to the local solution environment of small apolar solutes were identified at the molecular level in Monte Carlo calculations on $[CH_4]_{aq}$ at 25°C. In ionic hydration studies, the nature of ion-water complexes in aqueous solution and the interface of these structures with bulk water was detailed for dilute aqueous solutions of the cations Li^+, Na^+, K^+, and the anions F^- and Cl^-. The calculated aqueous solution coordination numbers of Na^+ and K^+ were found to be distinctly different, and structural indices which figure into phenomenological theories of membrane permeability and ion selectivity were extracted from the simulation results. For hydrophilic solutes, a general procedure for the analysis of the structure of the statistical state of the system was developed and illustrated in a computer simulation of $[H_2CO]_{aq}$ at 25°C. This approach was found to resolve the logical solution environment of the various functional groups in clear structural chemical terms. The theoretical determination of solvation sites was shown to follow naturally from this procedure of analysis. The simulation results on this system provide the most detailed theoretical view yet obtained of the local solution environment of the carbonyl group in liquid water. This work is being extended to provide a description of the local environment of all biomolecular functional groups in aqueous solution.

In addition, the first fully ab initio calculation of the free energy and entropy of liquid water was reported from this laboratory. A significant advance in the methodology for computer simulations based on the cavity-biased algorithm has also been described and documented. A calculation of the solvent averaged mean force between two methane molecules in aqueous solution (a computer simulation of a prototype hydrophobic binding situation) has also been completed and provides new theoretical data on the hydrophobic effect.

Dr. Beveridge is now extending his work to include interactions in aqueous solution via the potential of mean force, solvent effects on conformational stability via the potential of mean torsion and the organization of water in crystals of biological macromolecules.

2. Supersonic Spectroscopy
GM 25907-03 (Donald Levy), University of Chicago

Molecular spectroscopy is still one of the best and most direct ways of studying molecular structure. In effect, spectroscopy of this type looks at molecular motions--electronic, vibrational, and rotational. By analyzing these motions one can also see the flow of energy in systems undergoing a dynamic process, such as a chemical reaction.

Unfortunately, the multiplicity of spectral lines of even a diatomic molecule is so great that resolution is, under normal conditions, almost impossible. What one sees is a broad featureless band, rather than individual spectral lines. This can, to some extent, be overcome by lowering the temperature to reduce the number of energy levels available, i.e., by reducing molecular motion. Of course, at the temperatures required (near absolute zero), virtually all compounds are in the solid state. Although spectroscopy of solids may be useful, it cannot provide information about the isolated molecule.

In order to look at "cooled" molecules in the gas state, Dr. Levy has used supersonic molecular beams. Molecules are mixed with a monoatomic carrier gas and shot at high pressure through a small orifice into a vacuum. As the gas expands, and as it collides with the carrier, all the molecular motions are cooled to near zero degrees Kelvin. When tunable dye lasers are used, highly resolved molecular spectra can be obtained.

Dr. Levy has been examining the supersonic spectra of such biologically important compounds as phthalocyanins and porphyrins. Of particular interest is the capability of studying energy transfer between chromophoric side chains and the porphyrin ring. The studies carried out to date show that highly resolved spectra can be obtained even for such relatively large molecules. The information that is being obtained will provide fundamental insights into molecular structure and dynamics.

3. Use of Raman Spectroscopy for Determination of Conformations in Proteins GM 15547-13 (W. L. Peticolas), University of Oregon

Raman spectroscopy is a technique which measures the frequencies of molecular vibrations in molecules. In recent years, there have been a large number of studies of the Raman spectra of proteins and polypeptides in solution. Most of the observed Raman bands arise either from the molecular vibrations of the polypeptide backbone chain or from the amino acid side chains. Of particular interest is the fact that certain of the backbone chain vibrations change their frequency with changes in protein conformation. This seems especially true of the amide III C-N vibrations. Qualitatively, changes in α, β , and random coil can be related to intensity shifts in the location of the amide III band. Dr. W.L. Peticolas, following up some observations of Dr. R. Lord, has attempted to quantitate these shifts to more accurately follow changes in protein conformation.

The key observation was that the changes in the frequency of the amide III band appear to correlate with changes in the Ramachandran ψ-angle. Thus, it was proposed that the intensity in amide III region between 1230-1300 cm^{-1} is a histogram of the number of amide residues in the protein having ψ-values between 150° and -90°. (The Ramachandran $\phi - \psi$ angles can completely define a peptide configuration.)

First, the assumption is made that the ϕ angle has a relatively minor effect on infuencing the change in Raman frequency on going from one conformation to another. Then, the Ramachandran angle ψ was divided into four equal regions of 90° each, and Raman frequencies of the amide III band ranging from 1210-1300 cm^{-1} were mapped into these regions. Each region corresponds, roughly, to a different type of backbone conformation. A similar map of conformation vs. angle was done for the ϕ value. Thus, a 2-dimensional 4x4 chart can be constructed. This is composed of 16 squares, each having a different member of the pair ϕ-ψ.

Peticolas and his co-workers then took the Raman spectra of four proteins in H_2O and D_2O. The deuterated proteins will have only the peptide spectrum altered, since the vast majority of the side chains are not affected by deuteration. ϕ-ψcharts were made for four proteins, using known crystallographic coordinates, and compared to the experimental Raman frequencies. For lysozyme, the Raman intensities provide an almost exact prediction of the angles they

are assumed to reflect. However, for the other three proteins, the Raman intensities predict a smaller value for the number of β residues, and a larger value for β-turn residues, than is actually found. This appears to be due to a dependence of the Raman frequencies and intensities on the ϕ angle as well as the ψ angle. This can be readily corrected. However, more studies must be done before the correction process can be guaranteed.

Until further results are available, the full value of this approach cannot be completely assessed. However, it does appear to hold promise for evaluating conformational changes in proteins in solution. It has the potential of being a significant tool in our study of native protein structure.

4. Structural Studies of Dehydrogenases and Lipoproteins
GM 13925-14 (Leonard J. Banaszak), Washington University

In this work, two forms of the enzyme malate dehydrogenase are being investigated by single crystal X-ray diffraction analysis. The two forms are found in different compartments of cardiac muscle cells. One form, "soluble" malate dehydrogenase (sMDH), is always found in the cytosolic compartment. The second form (mMDH), is found in the inner membrane matrix of mitochondria. The molecular structure of sMDH has already been determined by Dr. Banaszak and that of mMDH is now underway. In the molecular model of sMDH, the coenzyme NAD+ is bound in a large cavity near the center of a protein subunit. Most of the noncovalent contacts between the coenzyme and substrate, and the sMDH are known. Such "contacts" (one atom to another) within or between molecules are the key energy factors in the recognition and chemical processing of biological metabolites.

The determination of any protein structure is an arduous task. It includes a series of steps: (1) purification and crystallization of the protein, (2) data collection from the native form, (3) determination of phase relationships for the native data, utilizing data from heavy atom derivatives, (4) calculation of electron density maps and atomic positions, and (5) structural refinements. The third step of this procedure is especially formidable in the case of mMDH, due to the occurrence of polymorphic crystal forms, especially with heavy atom binding. Dr. Banaszak has made impressive progress in solving these difficult problems, and should soon have atomic coordinates for mMDH.

This molecular model can then be carefully compared with that of sMDH. Knowing the molecular structure of the active sites of these related enzymes will be a vital factor in determining how the catalytic reactions work and are modulated in different ways in the two enzymes. A comparison of the molecular structures of sMDH and mMDH will also be an important step in determining the molecular basis of organelle formation and stabilization.

Turning to mMDH, the long-range biochemical significance of the structural study is threefold. First, it will provide a key structural example of a protein which may have been introduced into eukaryotic cells by the initial presence of a microorganism. A comparison of the structure of sMDH and mMDH is particularly interesting since it will be possible to study the molecular structure of the nucleotide binding and catalytic domains of both proteins. If the catalytic domains were from two different dehydrogenases, the structure around the so-called catalytic domain might be notably different. Such is the

case when sMDH is compared with glyceraldehyde 3-phosphate dehydrogenase. Although both sMDH and mMDH have an identical catalytic function, potentially large differences in their molecular structures are likely because of their different cellular location and "heritage".

Closely linked to the structural considerations will be the ability to semi-quantitatively account for some of the catalytic properties of the two related enzymes, properties which are different in sMDH versus mMDH. Although the X-ray structure only describes a static state, evidence already obtained with sMDH suggests that a complex conformational change in the polypeptide chain accompanies the binding of the coenzyme, NAD+. When similar data is obtained for mMDH, it should be feasible to account for measurable differences in Michaelis and equilibrium binding constants for the two enzymes. This is the type of comparison which could lead to a close link between structure and function.

Finally a careful examination of surface residues on the two malate dehydrogenases will be made to search for structural factors linked to organelle formation. Similar studies of another mitochondrial protein, cytochrome c has suggested that patches of basic residues account for surface binding to mitochondrial lipid bilayers. The sMDH, mMDH structural studies would provide more convincing evidence for this hypothesis. Regions of concentrated basic residues should be observable on mMDH, and absent in the corresponding region of sMDH. In addition, accumulating structural evidence on enzymes shows that directional access by substrates to the active site is limited. Surface binding to lipid aggregates such as the mitochondrial inner membrane matrix must occur in a manner which does not obstruct the substrate and coenzyme binding pathway. Such steric studies will be made on the molecular structures of sMDH and mMDH.

Before any of these comparisons can be made, the location of more than 6000 atoms for each protein must be determined. The X-ray studies supported by this grant are aimed at obtaining these coordinates and answering these evolutionary and structure-function questions. Since these and other enzymes play such a critical role in cell function, the molecular structures are one of the most important elements in understanding both the catalytic reactions and the question of how these chemical events are modulated in different compartments of the cell.

6. Structure and Function of Heart Membrane Proteins
GM 20379-07 (Bernard L. Trumpower), Dartmouth College

The objective of much research in bioenergetics is to understand how electrons are transferred through the cytochromes of the respiratory chain and how this electron transfer brings about the outward transport of protons across the mitochondrial membrane. The cytochrome $\underline{b}$-$\underline{c}_1$ segment of the electron transfer chain has proven especially suitable for such experiments. It is a lipoprotein assembly of two $\underline{b}$ cytochromes, $\underline{b}$-562 and $\underline{b}$-566; cytochrome $\underline{c}_1$, an iron-sulfur protein; and ubiquinone. This respiratory complex accepts electrons from various respiratory chain dehydrogenases and passes these to cytochrome $\underline{c}$. During this electron transfer, four protons are transported outward by the $\underline{b}$-$\underline{c}_1$ complex for each two electrons transfered to cytochrome $\underline{c}$. Efforts to exploit the $\underline{b}$-$\underline{c}_1$ complex for studying the mechanism of electron transfer and proton translocation are highlighted by the work of Bernard Trumpower, of Dartmouth College (GM 20379). Dr. Trumpower's approach has been to resolve the individual functional components of a lipoprotein fragment from the inner mitochon-

drial membrane called succinate-cytochrome c reductase. Electron transfer reactions in the b-c_1 complex are examined in the presence and absence of the resolved components. Using this approach he recently purified, for the first time in an active form, the iron-sulfur protein of the b-c_1 complex form from heart mitochondria. The successful solution and reconstitution of the iron-sulfur protein has established that this protein is required for electron transfer in the b-c_1 region of the respiratory chain and has permitted formulation of a working hypothesis regarding the mechanism of proton translocation.

When Dr. Trumpower examined which electron transfer reactions involving the b-c_1 complex take place in the presence and absence of the iron-sulfur protein, it appeared that the function of the iron-sulfur protein and the mechanism of electron transfer could be explained by a sequence of the type....succinate succinate dehydrogenase > ubiquinone > cytochrome b-566, b-562 > iron-sulfur protein > cytochrome c_1 > cytochrome c. Thus, in these experiments the iron-sulfur protein was required for electron transfer from succinate to cytochrome c and from ubiquinone to cytochrome c, but it was not required for electron transfer from succinate or ubiquinone to the b cytochromes. On the basis of these and other experiments, Dr. Trumpower proposed that electron transfer through the b-c_1 complex takes place by a cyclic mechanism, referred to as the protonmotive Q cycle, and that the iron-sulfur protein fulfills a uniquely important function in the proton translocating reactions required for energy conservation by oxidizing ubiquinone at the cytoplasmic surface of the inner mitochondrial membrane.

An important requirement of the postulated protonmotive Q cycle is that there must be thermodynamically stable forms of ubisemiquinone involved in electron transfer in the b-c_1 segment. In collaboration with Tomoko Ohnishi of the University of Pennsylvania (GM 12202), Dr. Trumpower discovered two such species of stable ubisemiquinone in isolated succinate-cytochrome c reductase. This is the first demonstration of stable semiquinones in an isolated respiratory chain complex and lends credence to the possibility that such species participate in respiration.

Dr. Trumpower's most recent progress toward establishing that the iron-sulfur protein of the b-c_1 complex participates in oxidation-reduction of ubiquinone is the discovery that a synthetic analog of ubiquinone, UHDBT, inhibits respiration by binding to this iron-sulfur protein. Insight into the mechanism of this inhibition came from the finding that this inhibitor has the same effects on electron transfer reactions in the b-c_1 complex as does removal of the iron-sulfur protein. Thus, in the absence of antimycin, UHDBT prevents reduction of cytochrome c_1 by succinate or ubiquinol, but has no effect on reduction of the b cytochromes; while in the presence of antimycin, UHDBT prevents reduction of the b cytochromes and of c_1.

By combining the use of this inhibitor with the recently developed methods for resolution and reconstitution of the iron-sulfur protein Dr. Trumpower showed that the amount of UHDBT required to inhibit respiration is proportional to the amount of iron-sulfur protein present. In addition, UHDBT changes the spectral properties of the purified iron-sulfur protein and likewise those of the iron-sulfur protein *in situ*, thus demonstrating that this ubiquinone analog binds to the iron-sulfur protein.

UHDBT is the first example in which an inhibitor of respiration has been shown to act directly on an iron-sulfur center. It is also the only example to date of a respiratory chain inhibitor which acts on bacteria, yeast, and plants, in addition to mammalian mitochondria. This provides an opportunity to test the possible universal function of iron-sulfur protein: quinone interactions in the respiratory chain and photosynthetic electron transfer chains of these species. In bacteria and yeast UHDBT is effective on whole cells, which may permit selection of structural mutants with these species as an approach to further elucidate how this iron-sulfur protein interacts with quinone. In addition, the inhibitory properties of UHDBT are ideally suited to permit use of this compound as a ligand for affinity chromatography, thus making it possible to purify the iron-sulfur protein of the $\underline{b}$-$\underline{c}_1$ complex from a wide variety of species. The availability of an inhibitory analog of ubiquinone which binds specifically, reversibly, and with high affinity to the iron-sulfur protein of the $\underline{b}$-$\underline{c}_1$ complex and a methodology for purifying this protein in a reconstitutively active form provides a unique opportunity to elucidate the mechanism of electron transfer and energy conserving proton translocation in the cytchrome $\underline{b}$-$\underline{c}_1$ segment of the respiratory chain.

7. Identification of a Common Cofactor in Molybdenum-Containing Enzymes
GM 00091-35 (K. V. Rajagopalan), Duke University

Since the function of a living organism is dependent on a complex and extremely finely regulated series of chemical reactions, our understanding of human disorders has benefited greatly from basic research on the chemistry and biochemistry of individual molecules or groups of molecules. At times, it may require decades of research for those benefits to be realized. Dr. K.V. Rajagopalan of Duke University has been studying the biochemistry of metals for more than 20 years. His recent investigation of the processes involved in the uptake and utilization of molybdenum in animals has led to the identification of a cofactor which appears to be present in all molybdenum-containing enzymes except nitrogenase. A small molecule which non-covalently binds to the enzyme, this cofactor is required for enzymatic activity. Studies have shown that the molybdenum cofactor contains a reduced pterin ring as a structural component.

Dr. Rajagopalan has focused much of his work on a hepatic molybdoenzyme, sulfite oxidase. Sulfite oxidase, located in the intermembrane space of mitochondria, is involved in the metabolism of sulfur-containing amino acids, and probably has the dual role of detoxifying sulfite and providing sulfate to the organism. Dr. Rajagopalan's work on the molybdenum cofactor is especially interesting because of its possible relationship to human disorders. Sulfite oxidase deficiency during development leads to severe neurological abnormalities and mental retardation. Dr. Rajagopalan has been applying his expertise in basic biochemistry of sulfite oxidase to elucidate the molecular bases of disorders involving this enzyme. Inactivation of sulfite oxidase could be due to an alteration in, or absence of, the protein portion of the enzyme or the cofactor. In one type of sulfite oxidase deficiency, it has been shown through immunological techniques that the protein portion of the enzyme is absent while the cofactor appears normal. Recently, by assaying for active and inactive forms of the enzyme as well as for the cofactor, Dr. Rajagopalan and collaborators have identified patients apparently lacking molybdenum cofactor. In these cases plasma molybdenum levels are normal but molybdenum and molybdenum cofactor are not detectable in liver. Sulfite oxidase activity cannot be detected in liver and protein cross-

reacting with antibodies to the normal enzyme is not present. Xanthine dehydrogenase activity is absent in the liver, but the protein portion of the enzyme is apparently normal, based on antibody studies.

While it appears that the lack of molybdenum cofactor may be the primary deficit in these cases, the reason for the absence of the apoenzyme is not clear. It is possible that the primary genetic defect involves the protein portion of the enzyme. However, this seems unlikely, since in other cases of sulfite oxidase deficiency, patients lack the apoprotein but have normal levels of active cofactor. Furthermore, Dr. Rajagopalan has shown that another molybdoenzyme, xanthine dehydrogenase, is also inactive, while its protein portion appears normal. On the other hand, the absence of sulfite oxidase apoprotein could result from the lack of cofactor. The presence of cofactor may be necessary to induce synthesis of the apoprotein. If the apoprotein is synthesized, the absence of cofactor could cause an abnormal final conformation of the enzyme. With an altered structure, the molecule may be labile and quickly degraded by cellular proteolytic enzymes, or perhaps may not be recognized by antibodies to the normal enzyme.

These findings lead to further interesting questions concerning the role of molybdenum cofactor in human health. The source of the cofactor must be determined. Although it may be synthesized in the body, it is also possible it is a vitamin and therefore required in the diet. In either case, in disorders involving a deficit, supplying the cofactor to the patient may be an effective method of treatment. Although the patients that have been studied were devoid of cofactor and exhibited severe neurological and mental impairment, we must consider the effects of a reduced level of cofactor which perhaps could have only subtle, yet detrimental, effects on neurological and mental development. Since several sulfur compounds, including for example taurine and cysteine, are neuroactive, their levels may be exceptionally critical to normal neurological development.

Dr. Rajagopalan is continuing his studies on the molybdenum cofactor to address these and other questions. Central to its further characterization is the complete chemical characterization of the cofactor, an effort which is now being undertaken in his laboratory.

8. Control of the Pyruvate Dehydrogenase Multienzyme Complex
GM 06590-22 (Lester J. Reed), University of Texas-Austin

Biochemists have gained a great deal of insight into cellular function by studying individual enzyme-catalyzed reactions using isolated preparations of enzymes. Within the cell these enzymes are functioning in a complex environment of thousands of other proteins and small molecules, many influencing a particular reaction through control of substrates, products and direct activation or inhibition. Enzyme activities can also be controlled by extracellular effectors such as hormones. To ensure optimum efficiency it may be expected that some enzymes, for example those catalyzing a sequence of reactions, would be physically associated. To some extent, this is accomplished by organizing certain enzymes on cell membranes or within intracellular organelles. The fact that at least some soluble enzymes are organized in specific ways into functionally significant assemblages has been demonstrated by the isolation and characterization of multienzyme complexes. These complexes contain several tightly linked enzymes which

catalyze related reactions.

Dr. Lester Reed has concentrated his research on the study of three multienzyme complexes which catalyze the transformation of pyruvate, -ketoglutarate and branched chain -keto acids to acyl derivatives of coenzyme A. Each complex contains a decarboxylating dehydrogenase which delivers an acyl group of the decarboxylated substrate to the lipoyl prosthetic group that is covalently linked to a transacetylase. The transacetylase in turn transfers the acyl group to the coenzyme A. The resulting reduced dihydrolipoyl group is then oxidized and thereby regenerated by a third enzyme in the complex - dihydrolipoyl dehydrogenase. These complexes have been studied from E. coli and a number of mammalian sources. In eukaryotic cells the complexes are found in the matrix space of mitochondria. Individual enzymes of the complexes have been purified to homogeneity and active complexes have been reassembled in vitro from component enzymes.

Dr. Reed's recent efforts have been directed at characterizing the regulation of the mammalian pyruvate dehydrogenase complex. Reactions carried out by this complex are important because they play a role in the control of both energy metabolism and lipogenesis. The pyruvate dehydrogenase multienzyme system catalyzes a complex process linking glycolysis and the tricarboxylic acid cycle, two of the most central metabolic pathways. In adipose tissue, liver and mammary gland, a significant portion of the acetyl-CoA formed is used in the synthesis of fatty acids and sterols. The pyruvate dehydrogenase complex contains three enzymes, pyruvate dehyrogenase, dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase. Pyruvate dehydrogenase is a 154 K dalton tetramer composed of two subunits each with molecular weights of 36 K and 41 K daltons. Dihydrolipoyl transacetylase consists of a single 52 K dalton chain and dihydrolipoyl dehydrogenase is a 110 K dalton protein with two identical subunits and two molecules of flavin adenine dinucleotide. Each complex consists of a core of 60 dihydrolipoyl transacetylase chains. Tightly bound to this core are 20-30 pyruvate dehydrogenase tetramers and 5-6 dihydrolipoyl dehydrogenase dimers.

9. Of Mitochondria and Kidney Stones
GM 05919-23 (Albert L. Lehninger), The Johns Hopkins University

The flow of fundamental research on cell structure and function, while exciting and revealing, is often circuitous and uncertain in its terminus. An especially fine example of this process is the research conducted by Dr. Albert Lehninger at The Johns Hopkins University School of Medicine on the conversion of foodstuff energy into ATP by living cells. Dr. Lehninger observed that calcium ions inhibit the normal formation of ATP during oxidation of substrate by isolated mitochondria. It was discovered in 1961 in Dr. Lehninger's laboratory that calcium ions added to a suspension of respiring mitochondria disappeared from the medium and could be recovered within the mitochondria. It was concluded that the energy derived from oxidation of substrate was diverted from ATP formation and was used to pump calcium from the medium into the mitochondria. Dr. Lehninger was soon able to establish a quantitative relationship between the number of calcium ions accumulated and the number of oxygen atoms utilized during respiration. Moreover, he observed that simultaneously with the accumulation of calcium ions, there was also accumulation of phosphate ions from the medium. The amounts of calcium and phosphate accumulated in this way were so

large that insoluble calcium phosphate precipitated in the mitochondria. The precipitated calcium phosphate could be visualized inside the mitochondria by electron microscopy.

This observation suggested that the accumulation of calcium and phosphate by mitochondria might be the long-mysterious first step in the biological calcification of bone by boneforming cells called osteoblasts. The major mineral component of bone is a calcium phosphate salt called hydroxyapatite, a crystalline material that can be detected and identified very readily by X-ray diffraction analysis. Dr. Lehninger, in collaboration with Dr. Aaron Posner of The Hospital for Special Surgery in New York, established in 1964 that the form of calcium phosphate accumulated by the mitochondria was not hydroxyapatite but some other calcium phosphate salt that was completely amorphous and showed no crystalline order. This was a rather unexpected finding, since merely mixing calcium and phosphate in the test tube can produce crystalline hydroxyapatite in a short time. Nevertheless, when calcium and phosphate were accumulated by mitochondria they failed to crystallize, and it was reasoned that the mitochondria contained some factor that could prevent crystallization of calcium phosphate.

Land and brackish water crabs salvage the calcium of their shells and store it in the liver temporarily as they undergo moulting, the loss of the old shell and the formation of a new, larger one. The stored calcium is then brought back via the blood to the newly forming shell, enabling it to harden within a day or two to protect the crab from its predators. Since the amount of calcium in the shell of a mature crab is quite large and most of it is temporarily stored in the liver as a calcium phosphate salt, Dr. Lehninger reasoned that the liver of the crab might contain large amounts of the calcification inhibitor.

Crab liver was found to be extremely rich in the crystallization inhibitor and thus proved to be a good source from which the factor could be purified. While the amounts of the natural inhibitor that could be prepared were still very small, use of NMR, mass spectrometry, and chromatographic comparisons with synthetic compounds led Dr. Lehninger to the probable identification of the inhibitor as 3-phosphocitric acid. Pure, synthetic phosphocitric acid proved to be an extremely potent inhibitor, far more potent than any other substance, biological or nonbiological, that had ever been found to inhibit growth of hydroxyapatite crystals. These results have led to the conclusion that phosphocitrate is probably at least one of the naturally occurring compounds preventing calcium phosphate crystallization.

In his most recent research, Dr. Lehninger and his collaborators have administered 3-phosphocitrate to experimental mice, rats, and dogs in which severe kidney calcification and urinary stones were induced, either by injection of excess calcium or by administration of large excesses of parathyroid hormone. When very small amounts of 3-phosphocitrate were injected into the animals at intervals, kidney calcification and urinary stone formation in these animals were completely prevented. Moreover, it was found that administration of 3-phosphocitrate did no harm whatsoever to normal calcification processes occurring in the long bones of the growing animals. Thus, phosphocitrate can prevent these pathological types of calcification. These findings therefore imply that a simple chemical compound, 3-phosphocitrate, might prevent abnormal calcification processes that occur in some kinds of kidney stones, in arteriosclerosis, and in liver damage caused by various drugs and industrial chemicals.

This long trail of research, extending over a thirty-year period and beginning with very fundamental pure research that at first appeared to have no medical relevance, has over a period of years led to newer insights and understanding of how abnormal or pathological calcification processes are kept in check by the normal organism. Moreover, these observations may conceivably become therapeutically useful in preventing some kinds of kidney stones. Chronic kidney stone formation is a serious condition that can ultimately lead to loss of renal function and destruction of the kidney. These investigations may lead to improved procedures for management of this painful and serious condition.

10. Cordycepin Analog of 2', 5'-oligo (Adenylate)
GM 26134-08 (Robert J. Suhadolnik), Temple University

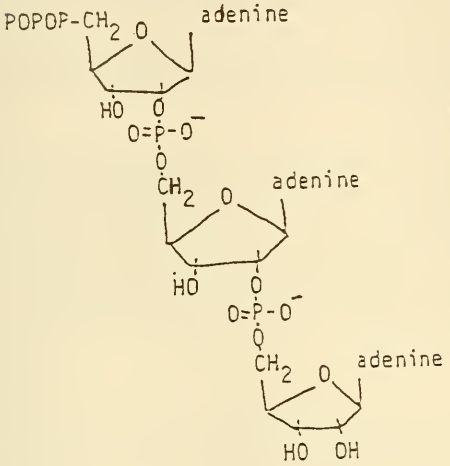
Interferons are glycoproteins secreted by virus-infected cells which enhance the antiviral resistance of other cells. Despite intense efforts, the molecular mechanisms of interferon action remain unclear. One response of a cell treated with interferon is an increased synthesis of certain enzymes, including a protein kinase, an endoribonuclease and an oligonucleotide synthetase. The synthetase catalyzes the formation of a series of oligonucleotides containing unusual 2' 5' phosphodiester bonds. These compounds are generically called (2'-5') pppA (pA)n (n= 1-10) and collectively referred to as 2',5'-oligo (adenylate). Levels of the synthetase can increase by as much as 10,000 fold after exposure of the cell to interferon. The 2',5'- oligo (adenylate) then activates a latent cellular endonuclease which degrades messenger RNA and decreases protein synthesis. It appears that this endonuclease attacks both viral and cellular messenger RNA. The action of 2', 5'-oligo (adenylate) is dependent on its continued synthesis, since it is rapidly degraded.

Recently, interest has grown in the possible use of interferon as an antiviral and antineoplastic agent. It has been difficult to obtain sufficient interferon for clinical use because it is present in very small amounts and interferons appear to be species- and perhaps tissue-specific. Nevertheless, major efforts have been made to obtain inferferon because of its exceptional promise as an effective therapeutic agent.

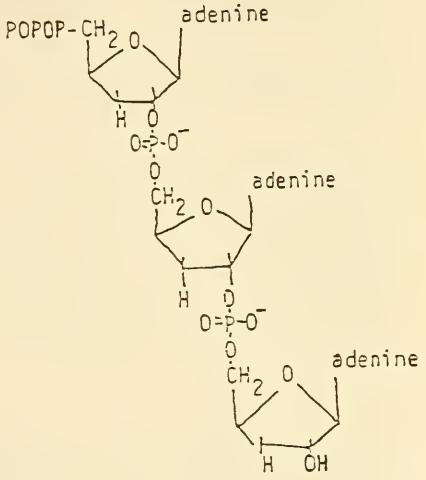
Since interferon causes several changes within the cell it may be possible to bypass the initial step and design compounds which affect events that occur later in the interferon sequence. In fact it has been shown that low concentrations of 2', 5'-oligo (adenylate) inhibit protein synthesis by activating an endonuclease which degrades both cellular and viral RNA. In designing such drugs their potential benefit as inhibitors of viral expression must be weighed against damage they may cause to the host cell.

Dr. Robert Suhadolnik has been concerned with the biosynthesis and use of nucleoside antibodies for many years. In 1965 he was first to report that a 2', 5' phosphodiester bond was naturally occurring in mammalian cells. Recently he has directed his research efforts to synthesize an analog of 2', 5'-oligo (adenylate) that would inhibit protein synthesis but have a longer half-life than the native compound. He has succeeded in synthesizing a cordycepin analog of 2', 5'-oligo (adenylate) that is 4-100 times more potent than the native compound. Furthermore, this compound is not degraded in HeLa cell extracts while the naturally occurring compound has a half-life of only 2.5 minutes. Dr. Suhadolnik has demonstrated that, like 2', 5'-oligo (adenylate), the cordy-

cepin analog can replace human fibroblast interferon in preventing the transformation of human lymphocytes following infection with Epstein-Barr virus. Also, like the naturally occurring compound, the analog is taken up into the cell and acts on an intracellular compound. A most encouraging aspect of this component is that while the naturally occurring 2', 5'-oligo (adenylate) is cytotoxic to uninfected lymphocytes and proliferating lymphoblasts, the cordycepin analog is not cytotoxic.



2',5'-oligo(adenylate)



cordycepin analog of 2', 5'-oligo (adenylate)

These studies raise the exciting possibility that structurally modified analogs of 2', 5'-oligo (adenylate) may be potentially useful in the treatment of human viral infections and human cancers.

11. Ornithine Decarboxylase and Cell Growth Control
GM 18538-11 (Glenn Kuehn), New Mexico State University

Ornithine decarboxylase (ODC) catalyzes the first, rate-limiting reaction of polyamine biosynthesis, in which ornithine is decarboxylated to form the diamine, putrescine. Much experimental evidence has established that a rapid increase of ODC activity is an invariant element in the response of all eukaryotic cells that are stimulated to proliferate. Scrutiny of this phenomenon has identified two properties which make ODC a unique representative of eukaryotic enzymes. First, a great number of diverse agents have the capacity to produce marked stimulation of the enzyme activity. These agents include tumor-promoting agents, viral transformations, hormone administrations and mechanical growth stimulation. Conversely, other reagents such as polyamines and interferon rapidly inactivate ODC in a variety of cell cultures. Second, the shortest apparent half-life of

any known eukaryotic enzyme, about 10 minutes, has been ascribed to ODC. Moreover, fluctuations in such ODC enzymatic activity need not involve new enzyme synthesis or metabolic turnover by synthesis and degradation. This hints of an enzymatic process involving post-translational modification.

The possibility that post-translational modification of ODC occurs was further strengthened when Heller, et al., subsequently discovered a protein factor of M_r 26,000 which complexed noncovalently with ODC and noncompetitively inhibited its enzymatic activity. The polyamines, putrescine, spermidine, and spermine appeared to induce the synthesis of the M_r 26,000 inhibitor protein. The inhibitor was designated ODC-antizyme. The occurrence of ODC-antizyme appeared to be ubiquitous. It was detected in every eukaryotic cell line tested, in prokaryotes, in rat liver, and Physarum polycephalum.

In previous work Dr. Glenn Kuehn's laboratory at New Mexico State University at Las Cruces reported the discovery and characterization of a new type of protein kinase that was polyamine dependent. This polyamine-dependent protein kinase of subunit M_r 26,000 catalyzed phosphorylation of a unique, acidic nucleolar protein of subunit M_r 70,000. The two proteins were copurified from phosphocellulose chromatography. Phosphorylation of the M_r 70,000 nonhistone protein by the polyamine-dependent protein kinase yielded a phosphoprotein which demonstrated numerous properties concordant with a specific regulatory role in rRNA gene transcription.

Drs. Kuehn and Atmar have shown that this nonhistone nucleolar protein is ODC. Moreover, phosphorylation of ODC by the polyamine-dependent protein kinase of M_r 26,000, sharply inhibits decarboxylation activity. This unique control mechanism represents a newly recognized example of control of the first enzyme in a biosynthetic pathway by a protein kinase that is in turn modulated by the end products of the respective pathway.

Thus, drawing on their previous work, ODC appears to be a multifunctional protein depending on its existence in a dephosphorylated or phosphorylated form. Dr. Kuehn's lab had previously detected a phosphoprotein phosphatase activity in nuclei and nucleoli of P. polycephalum which dephosphorylated phosphorylated ODC. In its dephosphorylated form, ODC functions as the catalyst for which it is named. In its phosphorylated form, ODC does not catalyze decarboxylation of ornithine, but demonstrates numerous properties which argue for a specific regulatory role in rRNA gene transcription in the nucleolus. Thus the phosphorylated ODC is a component of a deoxyribonucleoprotein complex isolated from nucleoli that contained palindromic ribosomal DNA. The purified phosphoprotein binds with high specificity to a region of purified ribosomal DNA near the symmetry axis of the palindrome. It stimulates rRNA synthesis by RNA polymerase I within the deoxyribonucleoprotein complex. Both binding and rRNA stimulation are dependent on the phosphorylation state of ODC. Therefore, it seems most likely that the phosphorylation of ODC serves an even broader role than preventing and controlling the biosynthesis of the polyamines.

Several pharmacological agents can rapidly inactivate ODC in mammalian cells on a temporal scale similar to that of the polyamines. These include cytochlasin B, colchicine, retinoic acid, and interferon. It is Dr. Kuehn's intent to determine whether any of these compounds exert their respective inhibitory effects through the action of the polyamine-dependent protein kinase on ODC. In the case of the

interferon effect on mammalian cells, the facts are particularly suggestive. Interferon induces a protein kinase activity which phosphorylates a polypeptide of approximately M_r 70,000. The M_r 70,000 polypeptide copurifies with the kinase. The appearance of the 70,000-M_r phosphoprotein is the most apparent difference between interferon-treated and control cell fractions, and it can serve as a marker of interferon's action. It appears only under conditions where the antiviral state is induced. Dr. Kuehn is currently collaborating with another investigator to determine whether ODC and ODC kinase are target proteins for interferon action. Their preliminary experiments support this hypothesis.

12. Role of Actin in Cell Motility as Evidenced by the Action of Cytochalasin B
GM 22289-07 (S. Lin), The Johns Hopkins University

Cell motility, a general term used to describe the movement of cellular or intracellular material, is a fundamental property of living cells. Included under this general terminology are cell locomotion, cell division, cytoplasmic streaming, endocytosis, exocytosis, etc. Although these phenomena have been studied for years, the molecular basis of cell motility has not been defined. Dr. Shin Lin's laboratory at The Johns Hopkins University, Baltimore has been studying the molecular aspects of movement and has used the cytochalasins in this research. The cytochalasins are a group of drugs discovered in the late 1960's which are metabolites produced by certain fungi. These substances have been shown to inhibit various kinds of cell motility in virtually all animal and plant cells tested.

Dr. Lin has used the drug Cytochalasin B (CB) extensively. This particular cytochalasin has the advantage of producing change rapidly in cells (effects are observable within minutes) and allowing cells to resume motile functions once it has been removed from the in vitro system. The mode of action of the drug has been elusive. About five years ago, Dr. Lin's research group began a systematic investigation on the mechanism of CB's effect on cell motility. The first step involved the synthesis of a radioactive derivative of the drug, tritium labeled dihydrocytochalasin B (^{3}HCB). This derivative was free of the unwanted side effects on membrane transport. This was important since CB inhibits not only cell motility but sugar transport into the cell, with the two properties believed to be independent of one another. Using the ^{3}HCB as a tracer the investigators were able to isolate a complex of proteins containing a high affinity binding site for the drug. The first isolation of this complex was achieved using human erythrocyte membranes, and this complex contained actin, spectrin and other minor components. Later studies resulted in the isolation of similar protein complexes from other cell types (human platelet plasma membranes and chromaffin granule membranes).

When the purified cytochalasin binding complex is added to a solution of actin (G actin) in a low ionic strength medium, there is a rapid increase in viscosity to a level comparable to that of a solution of filamentous actin. Electron microscopic studies of this viscous solution also revealed a similarity to filamentous forms of actin. The rate of induced actin polymerization was dependent on the amount of complex added to G actin.

Binding studies indicated that upon polymerization of the actin, the cytochalasin binding complex became associated with actin filaments. Low concentrations of

cytochalasins B, D and E and ^{3}HCB inhibited actin polymerization induced by the complex. The relative effectiveness of these drugs in inhibiting actin polymerization was correlated with their relative affinities for the complex as well as their relative potencies in affecting cell motility. These results suggested that the cytochalasin binding complex functions as a regulatory site for cell motility by controlling formation and membrane attachment of actin-containing microfilaments in the cell. The cytochalasin binding complexes induce the assembly of globular actin molecules into long filamentous structures resembling strings of beads. Their results indicate that cytochalasin B inhibits this type of filament assembly by binding to the growing end of the filament and blocking extension.

These studies correlating the action of cytochalasin B on actin filament assembly in vitro with the biological action of the drug provide good evidence that these filaments play a vital role in cell movements in non-muscle cells. One application to clinical medicine envisioned by Dr. Lin is the development of new therapeutic drugs which could block specific types of cell motility, such as the invasive movements of cancer cells.

13. Isolation and Localization of Asialoglycoprotein Receptor
GM 27989-02 (Harvey Lodish), Massachusetts Institute of Technology

A major discovery of the past decade has been the prevalence and variety of membrane receptors. Membranes serve as barriers between a cell's internal and external environments. They also are the locus for much of the sensory information - telling the cell what is in the surround. Membrane receptors detect molecules in the extracellular fluid and subsequently initiate intracellular signals, or they may transport molecules into the cytoplasm where these molecules may in turn control some cellular process, or even serve as a substrate. In either case, the receptors may bind with very high affinity and remarkable specificity. Even in specialized cells the receptors may be a scarce species, constituting a small fraction of membrane proteins.

Only a very few membrane receptor proteins have been purified to homogeneity. Although the asialoglycoprotein receptor from hepatocytes has been much studied, until recently there had been no complete purification. This protein is a major hepatocyte cell surface protein. It binds circulating plasma asialoglycoproteins (distinct from the normal sialoglycoproteins) and initiates translocation into intracellular compartments. Many questions remain about the role of the receptor, especially in translocation.

Recently Drs. Alan Schwartz and Harvey Lodish and their colleagues have succeeded in purifying the receptor upon solubilization in Triton X-100 and affinity chromatography. Four distinct polypeptides, of 40,55, 65 and 100-120 kD were isolated. A monoclonal antibody was prepared which both immunoprecipitates solubilized receptor activity and blocks the binding of galatose-terminal glycoproteins to immobilized receptor. The antibody immunoprecipitates all four polypeptide species. Moreover, peptide fingerprinting of the individual species demonstrated an identical pattern. These surprising findings raise the question of the nature of the differences between the species. The best guess at present is that different sugars are attached to the polypeptide moiety of the glycoprotein receptor. Another question is whether more than one species actually exists on the cell surface. To answer this question, the surface of intact hepatocytes was labeled

with ^{125}I. Membrane solubilization and immunoprecipitation with antireceptor antibody revealed two major bands at 55 and 65 kD. Thus, more than one species is present at the membrane surface in vivo. The ability of the investigators to surface label with ^{125}I and selectively immunoprecipitate allows them to quantify the surface receptor independent of ligand binding. They calculate that there are 500,000 functional surface receptor molecules per hepatocyte, constituting about 0.5 percent of the plasma membrane protein. This is the first surface receptor protein quantified independent of ligand binding.

The purification and isolation of a monoclonal antibody to asialoglycoprotein receptor will allow marked advances in the near future. To give one example, antibody is being coupled to various electron-dense markers. This will allow study of the topology of receptor distribution in the membrane and its biosynthesis, cycling and ligand transport. Evidence from the most recent findings already has lent considerable weight to the idea that receptors are internalized with the ligand, to be subsequently reinserted in the membrane. Isolation of other hybridoma clones may allow more reliable and detailed following of the receptor, in all cases allowing us to follow the antigen regardless of the state of the binding site.

14. Mechanism of Protein Synthesis on Rough Endoplasmic Reticulum
GM 21971-07 (G. Kreibich); GM 20277-09 (D. Sabatini), New York University

Transfer of secretory proteins into the lumen of endoplasmic reticulum (ER) and insertion of transmembrane glycoproteins into membranes are cotranslational processes. That is, it appears that the processes require the binding of ribosomes synthesizing those polypeptides to particular receptor sites in the membrane surface. Peptide insertion into or transit through the ER membrane occurs during synthesis. The specificity of the ribosomemembrane association is thought to be determined by information contained in a discrete segment at the amino terminal end of the nascent polypeptide chain which serves as an insertion signal recognized by specific membrane components. Much of these initial findings are the work of Sabatini (GM 20277) and Blobel (GM 27155).

During their passage across the ER membrane, secretory and membrane polypeptides may undergo cotranslational modifications, including removal of the signal segment by a membrane associated peptidase, covalent linking to oligosaccharide chains transferred from lipid donors in the membrane, and hydroxylation or other changes which are carried out by enzymatic systems located near the ribosome-membrane junction.

Drs. Kreibich and Sabatini have identified membrane proteins found in rough microsomes, but not smooth ones, which appear to be related to membrane bound ribosomes. These proteins, called ribophorin I and II, are present in a fixed stoichiometric ratio to the ribosomes. Treatment of intact microsomes with low concentrations of glutaraldehyde or with reversible bifunctional reagents leads to crosslinking of ribophorins to membrane bound ribosomes. Antibodies raised against the ribophorins from rat liver cross-react with the corresponding proteins identified in rough microsomes from other organs and species, indicating that ribophorins are a characteristic feature of all rough ER, as would be expected for proteins with an important function in the assembly of membrane bound polysomes. This is consistent with the ability to translate membrane or secretory proteins in mixed systems, with ER from a different source than the ribosomes

or message.

In vitro systems which affect the translocation of secretory proteins across the microsomal membrane have been developed. After mild proteolysis in high salt, dog pancreas membranes lose their ability to carry out vectorial translation (transmembrane secretion during translation). Incubation of these microsomes with peptides released during the proteolysis restore the translocation activity. The ribophorins are the leading candidates for these peptides.

Although the evidence is not yet definitive, there is a strong indication of the intimate involvement of ribophorins in ribosome-ER binding and subsequent translation and vectorial discharge. The investigators' working hypothesis is that ribophorin I represents the receptor for the insertion signal of proteins made on bound polysomes. Ribophorin II appears to be tightly associated with ribophorin I and may participate in one of the other processes associated with vectorial discharge or with cotranslational modification of newly synthesized polypeptides. Another cytoplasmic factor, of 93K daltons, appears to have affinity to both the N-terminus of secreted peptides and the ribophorins.

The major directions of this research in the near future are: ribophorin and 93Kd protein purification; raising of monoclonal antibodies to test for selective inhibition of the vectorial transport and synthesis, as well as to delineate the topology of the protein distribution in the rough ER; reconstitution of membranes able to bind ribosomes and translocate proteins during synthesis; cloning of cDNA's from ribophorin I and II messengers, the ultimate aim being to sequence the messages and hence the protein; and, finally, identification of the segments of the ribophorins imbedded in ER membrane and those responsible for other functions such as ribosomal binding.

15. Pathways of Membrane Biogenesis
GM 26755-03 (Randy Schekman), University of California, Berkeley
GM 25662-04 (James Rothman), Stanford University

Among the major questions in cell biology are the synthetic pathways and the mechanisms of morphogenesis of organelles. The proteins of cell membranes are often synthesized on the rough endoplasmic reticulum. They must then be transported to the cell surface, incorporated into or associated with a membrane bilayer, and, if glycoproteins, glycosylated. The synthetic sequence and cellular route resulting in the insertion of a membrane glycoprotein into plasma membrane have long been unknown. Recently several laboratories have developed systems to attack these questions. Two labs which have had marked success are those of Randy Schekman (GM 26577), University of California at Berkeley, and James Rothman (GM 25662), Stanford University. Using two very different approaches, they have been able to learn a great deal about membrane biogenesis in eukaryotes.

Dr. Schekman has isolated a series of yeast secretory (sec) mutants that are temperature-sensitive for cell surface, division, and secretion. Most of these mutants accumulate secretory proteins in an intracellular pool which can be released when cells are returned to a permissive temperature. At least 23 gene products have been implicated in the process of delivering membrane and secretory proteins to the cell surface. Electron microscopic analysis has revealed three distinct membrane-bound organelles that accumulate in different

mutants. These observations provide evidence of the enzymes, biochemical pathways and organelles involved in processing. The results obtained so far are consistent with the following model: Secretory proteins enter the endoplasmic reticulum (ER) where the initial steps of glycosylation occur. Nine or more sec gene products and energy are required to transfer material to a Golgi-like structure where further glycosylation occurs. Two or more gene products and energy are required to package nearly fully glycosylated proteins into vesicles, which are then transported into the plasma membrane where they fuse with the plasma membrane in a process that requires at least ten additional gene products and energy. Secreted proteins may be inserted into a bud which may pinch off as a vesicle. Plasma membrane growth just requires protein incorporation into the plasmalemma. There appears to be a pathway that allows some assembly to bypass this major pathway. Also, a branch in this pathway may contribute to the assembly of other cellular organelles, such as lysosome-like structures.

Dr. James Rothman has adopted a very different strategy. He has used mammalian cells, such as CHO and rat liver cells. In order to follow membrane glycoprotein synthesis he has synchronously infected the cells with vesicular stomatitis virus (VSV) whose envelope contains only one species of integral membrane protein, G protein. Like host-cell glycoproteins, G protein is transported from its site of synthesis and initial glycosylation in the ER to the plasma membrane via the Golgi apparatus. Rothman has found that the newly synthesized G protein could be transported in crude extracts of CHO cells from endoplasmic reticulum-derived membranes to membranes of the Golgi complex. Highly purified fractions of Golgi membranes can substitute for the Golgi of the infected cells but smooth ER cannot. This transport has also been shown to be energy-dependent. The evidence further suggests that the protein is transported in two waves and in two forms. The early wave involves transport of a high mannose G protein in clathrin-coated vesicles. This is presumably from ER. The second wave, also in clathrin-coated vesicles, is of a mature G protein, one to which terminal sugars, including sialic acid, are attached. This is presumed to be the transport of protein from the Golgi to the plasma membrane.

Two different studies--one using sophisticated genetic techniques to study yeast secretion and membrane growth; the other, mammalian viruses to observe the transport of viral coat proteins in cell extracts--have told us a great deal about membrane synthesis in normal eukaryotic cells. Many other laboratories are using similar systems to continue the work, and progress promises to be rapid over the next several years.

16. Establishment of Polarity and Development
GM 19363-18 (John Gerhart), University of California, Berkeley

The unfertilized egg of Xenopus laevis is radially symmetric about its animal-vegetal axis, while the later-stage embryo is clearly bilaterally symmetric with an anterior-posterior axis and a dorsal-ventral axis. Almost a century ago, Roux, Morgan, and Boring, and others observed that shortly after fertilization the uncleaved egg acquires bilateral symmetry when it forms the grey crescent, a less pigmented region on one side of the animal hemisphere opposite the side of sperm entry, and that the asymmetry of the egg in general has a precise relationship to the asymmetry of the embryo. That is, the lower limit of the grey crescent appears to coincide with the site at which the blastopore first appears a day or so after fertilization, and the crescent as a whole corresponds to the position

of eventual dorsal embryonic structures such as the notochord, somites, and neural tube. Since local insemination experiments demonstrate that the sperm can penetrate anywhere in the animal hemisphere, and that the grey crescent and dorsal structures invariably arise at a position opposite the point of sperm entry, it seems clear that the radially symmetric unfertilized egg has around its entire circumference the potential to become asymmetric under the influence of the sperm. Furthermore, it seemed clear that the early asymmetry committed the egg to a definitive pattern of regional developmental assignments expressed at later stages.

Later, in 1924, Spemann and Mangold discovered that the early gastrula of the newt contained in the region above the blastopore a small group of cells capable of organizing the development of dorsal structures from surrounding cells; for example, organizing an entire secondary embryonic axis when transplanted into a ventral equatorial position in a recipient early gastrula. Clearly the small region above the lip of the blastopore was a source of inductors which would "dorsalize" the embryo. The prospective dorsal-ventral axis of the early gastrula was defined by the position of this region. Since the organizer region occupied the equivalent position of the grey crescent, it seemed plausible that the organizer gained its special dorsalizing powers directly from the crescent itself or from the subadjacent cytoplasm. Thus, the definitive dorsal-ventral axis of the embryo appeared to originate by the action of the grey crescent, the position of which was fixed shortly after fertilization by the entering sperm.

Dr. John Gerhart and his coworkers at the University of California, Berkeley, have reexamined this "classic" picture and doubt its basic validity, at least for X. laevis. To be sure, they have established that the sperm entry point (SEP), grey crescent, blastopore, and dorsal embryonic side have the classic topographic relations in X. laevis, but the original evidence for the role of the grey crescent as the causative agent in dorsal localization is not supported by their recent work.

Dr. Gerhart, working with Drs. Kirschner and Uhbels, demonstrated that normal topographical relationships could be disrupted by gravity. They turned the egg "on the side," that is, inclined it 90° so that the animal-vegetal axis was horizontal rather than vertical. They could then check that the SEP was on the uppermost side of the inclined egg. In this position, the internal vegetal yolk mass slips downward slowly and the animal hemisphere contents move upward compensatorily. After 20 minutes in this position, the egg was transferred to dilute Ringer's medium where the perivitelline space inflated again and the egg returned almost to its original and normal upright position. After 9 hours the blastopore, to their surprise, invaginated on the same side as the SEP, not opposite it. Subsequent experiments showed that whatever position of the egg equator was uppermost in the 90° inclination, that region became the dorsal side of the embryo. Thus, gravity could easily overcome the influence of the sperm and determine the orientation of the egg's commitment to dorsal development.

When the same treatment was applied to eggs in the period after grey crescent formation, but before the first cleavage, the investigators found an increasing resistance of the egg to the axis orienting effects of gravity. Using a normalized time scale of 0 to 1.0 to represent the time period to first cleavage,

they found that at 0.7, the egg was completely resistant to a 20 minute treatment at a 90° inclination. This resistance can be partially overcome by a 60 or 90 minute treatment at 90°. They conclude that the position of the future dorsal side of the egg can be dissociated from the grey crescent by applying more force to rearrange internal materials of the egg and perhaps to form a cytoplasmic localization by gravity. Previous workers did not think the grey crescent could be separated from dorsalization.

Dr. Gerhart and his students have studied UV-irradiated eggs, which have been shown by others to cleave and gastrulate, but then fail to neurulate. They produce radially symmetric "ventral embryos" of the sort Spemann and Fankhauser produced by eliminating the prospective dorsal portion of the egg by ligation. It has been suggested that ventral development is a sort of "ground state" open to all regions of the egg equatorial circumference, but that dorsal development requires something special. UV is a convenient means to block the egg's mechanism for establishing a dorsal cytoplasmic localization. The egg is sensitive to UV until the time 0.6, when resistance appears. After 0.7 the egg is insensitive to UV in that dorsal development is normal. Further, Dr. Gerhart and his collaborators find that all UV-treated eggs can be completely rescued for dorsal development if they are inclined 90° for 30 - 40 minutes starting after the UV irradiation. Eggs rescued by a short gravitational treatment develop to embryos indistinguishable from unirradiated controls. The dorsal side of the UV-treated eggs always arises from the uppermost equatorial position. They also find that eggs of some UV-treated batches can be rescued by gravity even in the period after first cleavage. This is the first evidence that the eggs are still receptive or responsive to gravity-directed cytoplasmic reorganization at such a late time, long after grey crescent formation.

Thus these investigators conclude that gravity is able to induce an acceptable dorsal cytoplasmic localization in an egg unable to form its own. They believe that in using gravity to control the axis position in unirradiated eggs, especially in the post-crescent period, they must be inducing dorsal cytoplasmic localizations de novo by gravity, and not just shifting around a localization previously formed by the egg.

From the above evidence, Dr. Gerhart and his collaborators have proposed an alternative to the grey crescent-->dorsal localization theory. They propose that the egg cortex as a whole is a contractile organelle (an idea substantiated by their evidence, reported elsewhere, that the fertilized egg undergoes periodic cortical contractions). Their theory states that the cortex undergoes an asymmetric contraction toward the SEP side of the egg during a short time period about halfway through the interval from fertilization to first cleavage, and that it drives a rearrangement of sub-cortical vegetal cytoplasmic materials connected to the cortex.

Whatever the ultimate status of this theory, these investigators have brought about a major rethinking of the mechanism of developmental organization in the amphibian.

GOALS AND OBJECTIVES OF THE PROGRAM

The Genetics Program continues to be directed toward gaining a better understanding of the fundamental processes and mechanisms of inheritance in health and disease. The ultimate objectives of the research and research training that we support are the prevention, therapy or cure of human genetic disease. It should be emphasized that the program considers the term "genetic disease" to encompass a broad spectrum of conditions. The term, as we use it, includes not only those conditions with single gene (Mendelian) modes of inheritance (examples are cystic fibrosis, hemophilia and Huntington's disease) or chromosomal abnormalities (for example, Down's syndrome) but also more common degenerative conditions such as atherosclerosis and diabetes. The risk of occurrence of such conditions is determined by both genetic and environmental factors.

The NIGMS Genetics Program differs in a number of ways from numerous categorical (specific disease oriented) research support programs in other NIH institutes which are also concerned with such diseases:

a. A major component of research supported by the Program focuses on such fundamental topics as nucleic acid chemistry, the mechanisms of transmission and expression of genetic information, and population genetics. Model organisms such as bacteria, fruitflies, and mice are extensively employed in this type of research.

b. The program also emphasizes that genetics is not only a discipline but a mode of thinking about biology and medicine that complements the more specific, targeted approach demanded by other more categorical NIH programs. For example, the study of rare (e.g., Lesch-Nyhan disease) or more common but relatively benign (e.g., G-6-PD deficiency) conditions has often illuminated our knowledge of genetic disease far out of proportion to their intrinsic medical importance. In the future, disease models will be needed that may provide generalizable concepts applicable to common diseases as diverse as diabetes and schizophrenia.

In summary, the program considers itself as a resource for generating techniques and concepts that transcend concerns about specific genetic diseases. It seeks to accomplish its objectives by supporting research and research training in basic research in genetics. Characteristically, the preponderance of research that led to recombinant DNA technology, as well as much of the early developmental work, was supported by the Genetics Program. As the utility of these techniques to the analysis of biological and medical problems has become more apparent, support for investigators utilizing these methods has become more widespread among all of the NIH awarding units.

ORGANIZATION AND STAFFING

In the latter part of 1980, the Genetics Program of NIGMS was reorganized in order to better reflect significant new trends and perceived changes of emphasis in the field of genetics. The Program now consists of two sections: the Molecular and Medical Genetics Section and the Genetics of Growth and Differentiation Section.

The Molecular and Medical Genetics Section, was first led (in an "acting" capacity) by Dr. Fred H. Bergmann. In February 1981, Dr. David Beck was appointed Section Head of this group. In addition to Drs. Beck and Bergmann (who, besides his duties as Program Director, continues to administer a small portion of this section's portfolio), professional staff of the section includes Dr. George Woolley, Dr. Barbara Williams, and Dr. Irene Eckstrand. Dr. Eckstrand, who joined the Program in January 1981, is a visiting exchange scientist, under the Intergovernment Personnel Act, from the University of Texas at Austin. The Molecular and Medical Genetics Section supports research on topics such as the physical and organic chemistry of nucleic acids, mechanisms of replication, transcription and translation, medical and behavioral genetics, and cytogenetics and gene mapping.

Scientific developments in genetics have been unusually rapid in the past few years. New techniques, such as recombinant DNA technology, DNA sequencing methods, and assays using highly specific monoclonal antibodies, are leading to conceptual and technical advances in areas previously recalcitrant to molecular biology and molecular genetics. Amazing progress is now being made in areas as diverse as cellular differentiation, morphological development, and evolution, and commonalities among these processes are beginning to be discerned. In order to highlight these new developments, and to bring some organizational unity to research in this area, a second section, called the Genetics of Growth and Differentiation Section, has been established. Scientific fields supported by this section include chromosome organization and mechanics, developmental genetics, the control of gene expression, rearrangement of genetic elements, and the dynamics of genes in populations. The latter subprogram encompasses a number of topics in population and evolutionary genetics, and ecogenetics. The section is headed by Dr. Elke Jordan and includes Dr. Dorothea Miller and, in a part-time capacity, Dr. Carl Rhodes. Dr. Robert Gulley, who administered a portfolio of grants in the section, left in the summer of 1981 to begin law studies.

WORKSHOPS

1. Workshop on Mouse Mutagenesis, Bethesda, Maryland, February 4, 1981

Around November 1980, eight investigators involved in aspects of mouse genetics sent Dr. Bergmann a letter which suggested that it might be very worthwhile to undertake a cooperative search for mouse mutants. They felt that recently discovered unusual properties of ENU (ethyl nitrosourea) might bring such a search into the cost-effective range, and that this might provide opportunities for important research. The letter suggested that NIGMS convene a workshop to further discuss these ideas and--if they were considered feasible and important--how to bring them to fruition.

Workshop participants included four signers of the letter, who presented their point of view to the other attendees. Some of these other attendees were investigators chosen because of the breadth of their interests in biological or biomedical research, rather than for any specific expertise in mouse genetics. Dr. Susan Lewis, of Research Triangle Institute, was asked to attend to provide the others with up-to-date technical background on ENU mutagenesis in the mouse.

It was felt that the administrative and technical aspects of the proposed undertaking, which could be complex, and possibly costly, might well provide opportunities for constructive interactions between various funding units. For this reason a number of administrators from other agencies, such as the Department of Energy, EPA, and NSF, as well as from several NIH Institutes and Divisions, were invited. Many of these participants were extremely knowledgeable about mutagenesis, the mouse as an experimental animal, etc., and were a welcome addition to the group not only from the administrative point of view, but also because of their technical and scientific expertise.

A high level of enthusiasm was expressed throughout the group with regard to the promise of the ENU technology.

The participants discussed the relative value of developing mutations in whole mice, rather than in tissue culture. It was clear that there were many things that could not be done in tissue culture. Examples were mutations that affect development and specialized functions (hemoglobin, etc.), and, more generally, those mutations in which the relationship between the whole animal phenotype and defects demonstrable in cell culture is far from clear. It is relatively easy to develop a complementary tissue culture system from a mutant animal strain. Such complementary systems cannot generally (e.g., teratocarcinoma systems were discussed) be established starting with tissue cultures.

A number of important advances can be predicted as outgrowths of a systematic program in ENU mutagenesis:

a. The technology offers a quantum jump in our ability to understand the nature of gene regulation.

b. A mass mutagenesis program will generate enormous numbers of mutations which will contribute to the research of a great many investigators.

c. Through observing electrophoretic differences, recessive mutations can be identified in the heterozygote. Such identification will add an entirely new dimension to the elucidation of gene action. One clear benefit may be the recognition of mutant enzyme products associated with such genetic diseases as cystic fibrosis, Huntington's disease and diabetes; the proteins involved are unknown at present.

d. A related development could well be the production of mouse models of many human diseases, and perhaps even those whose molecular basis is at present poorly understood.

e. The number and variety of mutations identified will contribute enormously to the mapping of loci in the mouse genome. It was emphasized that the recognition of the potential of ENU mutagenesis comes at a time when the technology for freezing of mouse zygotes is becoming reasonably well established. The two developments complement each other.

The meeting adjourned with general concurrence on the value of this program and with a feeling of satisfaction that a start has been made.

A number of salutary developments since February 1981 can be traced directly to matters discussed at this meeting. At that time, the possibility of a concerted program to develop specific kinds of needed mouse mutants was discussed. The rather stringent fiscal situation since that time has made implementation of such ideas somewhat unrealistic. However, several of the participants at the meeting have begun mutagenesis programs utilizing ENU. This was a direct outgrowth of the enthusiasm generated because of the potential of this mutagen for readily developing useful mutants in the mouse, and was facilitated by the expressed willingness of personnel of Research Triangle Institute to expose males to the mutagen for breeding protocols to be done elsewhere. This has, in effect, reduced exposure of laboratory personnel to a rather dangerous carcinogen, by restricting its use to a locale that has developed experience in its handling. (The workshop participants were encouraged to develop a healthy respect regarding the hazards of working with ENU.)

In addition, a number of elegant ideas for better exploiting the potential of ENU as a mutagen in the mouse were first expressed at the conference. For example, a protocol for determining the optimal level of exposure to mutagen which would most efficiently permit detection of phenotypes only expressed in the homozygous stage (by determining the "break-even point" at which recessive lethals would reduce the yield of such mutants) was developed. Experiments along this line are developing, and will continue to develop, baseline data on which much future work with this new mutagen must depend.

2. Toward Implementation of the Concept of a Nucleic Acid Sequence Bank: Workshops, Briefing Sessions, Etc.

The rationale and need to develop a nucleic acid sequence data bank was been discussed in the Fiscal Year 1980 Annual Report (see p. 43). A major planning meeting, organized by Dr. Elke Jordan, Genetics Program, and Dr. Marvin Cassman, CMBD Program, was held in July 1980. Two additional technical workshops (October 27-28, 1980, and December 7, 1980) led directly to a number of developments. A letter to Science by Dr. Elke Jordan, (Science 210, 1074, December 5, 1980) acquainted the scientific community with overall plans for the bank, and invited participation in an interim sequence collecting effort. A "sources sought" announcement (NIH Guide to Grants and Contracts, April 2, 1981) mentioned our intent to seek contracts for both Project 1 ("the collection, verification, and initial distribution of nucleic acid sequence data...") and Project 2 ("software development for data base management, sequence analysis and manipulation, and user communication"). Draft "Requests for Proposal" were developed.

During the maturation of these ideas in the past year, it became abundantly clear that the type of activity envisaged would be quite costly, and could not possibly be borne by this Institute alone. For example, it was estimated that the project might cost about $2 million in the first year of operation, with gradually decreasing amounts thereafter, and that a commitment of five years of funding would be required to get the project started. NIGMS staff considered this situation as a challenge to develop a model for new, cooperative ways to fund important resources for biomedical science. Plans were therefore developed to seek financial contributions from the other NIH institutes and other Federal agencies. A briefing meeting was held at NIH on June 12, 1981, and was attended by representatives of most NIH Institutes and outside agencies such as DOE, DOD, NSF, and FDA. Subsequently, formal letters inviting participation were sent out. A number of funding agencies have responded with positive offers of support. As a result, NIGMS is proceeding with plans to announce the availability of "Requests for Proposals" in fall 1981, for evaluation and possible funding during Fiscal Year 1982.

3. Workshop on the Regulation of Protein Synthesis Through Covalent Modification, Bethesda, Maryland, January 18-20, 1981.

There were abundant reasons for convening a workshop on this topic at this time. The regulation of protein synthesis is an important aspect of many processes affecting the economy of a cell, including (1) viral takeover of the cell's translational machinery and, conversely, (2) establishment of the antiviral state by interferon, (3) the sequential and ordered expression of genetic information present in latent mRNA during embryonic development, (4) the rapid adjustment of protein synthesis to altered energy or nutritional status of the cell, and (6) coordination of processes such as ribosomal RNA and ribosomal protein synthesis, or of heme and globin biosynthesis in hemoglobin-forming cells.

The translational system is highly complex, and its activity appears to be sensitively and rapidly modulated. Generally, this occurs by covalent modification (for example, phosphorylation or dephosphorylation) of pre-existing components. Because of the complexity of the systems involved, scientific consensus and progress toward understanding has been slow. In fact, many aspects of covalent modification-control of translation are highly controversial, and the field has been in some disarray. This is a matter of appreciable concern to this Institute, which funds many of the important investigators in this field.

One of the chief purposes of the meeting was to encourage exchange of knowledge on the molecular events surrounding control reactions, to emphasize the criteria required to establish the causal relations between a modification reaction and possible physiological consequences and to stimulate exploration of covalent reactions other than phosphorylation in translational control.

The organizers felt that students of protein synthesis and its control could benefit from exposure to investigators of other systems where covalent modification leads to physiological control. Accordingly, the participants included representatives from two groups that do not frequently interact: (a) students of covalent modification controlling other systems such as coordinate metabolic pathways or cellular oxidation-reduction, and (b) students of translational control mechanisms.

The organizers of the workshop were Dr. Barbara Williams, Genetics Program, NIGMS, and Dr. Brian Safer, Acting Chief, Laboratory of Protein Synthesis, NHLBI. The effort was jointly funded by NIGMS, NHLBI, NCI, NIAMDD, and the International Union of Biochemistry. The meeting was attended by several hundred scientists from about 10 countries, and utilized the excellent, modern conference facilities of the Uniformed Services University for the Health Sciences. Presently, Dr. Safer is reviewing manuscripts from the participants for inclusion in one volume of the series "Current Topics in Cellular Regulation, (Academic Press, E. Stadtman and B. Horecker, Eds.).

CONTRACTS

The Human Genetic Mutant Cell Repository, Contract No. 1-GM 9-2101, The Institute for Medical Research, Camden, New Jersey

The Human Genetic Mutant Cell Repository (Cell Bank, for short) was established in 1972 with the philosophy of providing to the research community high quality, well characterized human cell lines, especially those from interesting but rare patients that might be available in only a few hospital settings. The goal of the Cell Bank is to stimulate research on human genetic diseases, rare or common, particularly by investigators who might have a hypothesis or a technique that could not be tested were cells not available from the Cell Bank.

Interest in, and activity of, the Cell Bank has remained high, and the most recent statistics show that 505 cell lines were received or expanded and nearly 3200 cultures distributed in 1980. The Cell Bank is actively pursuing previously initiated collections, principal among which is the psychiatric collection. From a meeting of nationally recognized consultants, advice was obtained as to the size and design of a collection of cell lines that would facilitate cellular research on psychiatric illness.

The Bank has been actively pursuing a collection of cell lines trisomic for each of the human chromosomes. Achieving such a collection appears to be impossible for biological reasons, so the Advisory Committee has recommended that the same result can be obtained by collecting lines having translocations distributed throughout those chromosomes (1, 3, 5, 6, 7, 11, 17, 19, 20, 22) not represented by the present collection. The trisomy collection is potentially a very useful collection for gene dosage and mapping studies, and it is thus being pursued vigorously.

Of major concern to the Cell Bank has been the collection and use of appropriately matched control cell lines. Since data on cell lines from various diseases are interpreted by comparison to a "normal line," selection of that control is exceptionally important. An appropriate control line is one for which there is good assurance that it is free of the disease under study. A line from an "apparently" normal family member may not be a legitimate control. Furthermore, the site of biopsy has a major effect on the nature of the cells represented in a fibroblast line. Papillary and reticular fibroblast subtypes grow differently out of different skin samples, and thus comparison of fibroblasts isolated from various body locations may be invalid. Finally, control lines are most often chosen for being "good growers." Cell lines representing disease states, however, are often not good growers, and thus a bias of results may be introduced. The issue of controls will be considered further by the Bank.

A number of other collections are also being expanded. These include the connective tissue diseases (especially osteogenesis imperfecta), neurological diseases (including Tourette syndrome), hemoglobinopathies, and X-chromosome abnormalities.

A major new initiative in ophthalmological disease has been started. There are approximately 250 inherited ocular disorders in five areas: retinal and choroidal diseases, corneal diseases, lens diseases, neurosensory diseases, and glaucoma. Significant opportunities are available in this field, and an expert panel will be convened in the fall 1981 to design this collection.

The curator system for providing oversight of the specific subcollections appears to be working well, and several new curators have been added. Dr. Irene Maumenee will monitor the ophthalmological collection, Dr. Barbara Migeon the X-chromosome collection, and Dr. Ara Tourian the neurodegenerative collection.

The Cell Bank may be viewed, at this point in its development, as a mature, although definitely not static, operation. Many of the collections that have been assembled over the years are reaching completion and are being described for publication. New collections are being added, but on a deliberate, rational basis after formal planning by the Advisory Committee. Use of the Cell Bank by the research community is increasing, and it appears to be firmly established as a research tool in human genetics. The Genetics Program is now exploring ways to encourage other parts of the NIH to consider the Cell Bank an indispensable adjunct to their efforts in genetics research.

RESEARCH HIGHLIGHTS

A. General. The Genetics Program of NIGMS continues to support much of the work at the leading edge of genetics research. Thus, we were pleased but not surprised that two of the three 1980 Nobel Prizes in chemistry were awarded to investigators funded by the program.

Half of the 1980 chemistry prize was awarded to Dr. Paul Berg of Stanford University. The Nobel Prize committee cited Berg for "his fundamental studies on the biochemistry of nucleic acids, with particular regard for recombinant DNA." He is recognized as the first investigator to construct a recombinant DNA molecule, i.e., a molecule containing parts of DNA from different species. Berg's work was thus a major contributing factor to the development of recombinant DNA technology.

For more than 20 years, Berg has been a leader in nucleic acid research and what is now called "molecular genetics." Starting in the late 1950's, his laboratory did much of the innovative work on the mechanisms of protein synthesis in bacteria. Later, his research turned toward the genetic structure of an animal virus, SV40, and the interaction of the virus with animal cells in culture. Berg should be particularly cited for responsible leadership in persuading scientists to declare a moratorium on certain recombinant DNA experiments until their risks could be assessed and guidelines developed.

The other half of the Nobel chemistry prize was awarded jointly to Dr. Walter Gilbert of Harvard University and Dr. Frederick Sanger of Cambridge University, England.

Like Berg, Walter Gilbert has worked for more than two decades on a wide variety of biological problems. He was the first to isolate and characterize a bacterial regulatory protein, the lac repressor. This protein is part of the genetic machinery that helps a cell to "sense" its environment (in this case, the presence of sugars such as lactose) and to respond by "turning on" certain genes to make enzymes (here, enzymes that break down lactose to simple compounds).

In more recent years, Gilbert developed one of the two methods now used for determining the sequence of nucleoside bases in DNA. Such DNA sequences are the ultimate "language of living things," and the ability to "read" them has added a necessary new dimension to understanding gene action. According to Gilbert, "DNA sequences are the basic, underlying structure. There is nothing more primitive. Your questions are ultimately posed there."

The work of NIGMS grantees, Berg and Gilbert, represents the peak of modern efforts in molecular genetics, providing new concepts of genome organization, and of how such organization leads to control of gene action. Such concepts are already having an important bearing on our ideas in fields as diverse as the mechanisms of genetic disease, cellular differentiation, and evolutionary theory.

Perhaps an even more profound aspect of Gilbert's and Berg's work has been its role in the development of important methodologies such as recombinant DNA technology and nucleic acid sequencing. Much of the work described in the following sections--isolating bits of genetic material, combining them with plasmid or virus DNA, transferring the resulting combination, i.e., recombinant DNA, into bacteria or mammalian cells, studying how such material acts in a new environment, and reisolating it and determing its sequence of building blocks--is the very fabric of modern genetics, and could not conceivably have been done even a decade ago.

This ability to isolate and purify genes and to study them in a variety of cellular environments has enabled molecular geneticists to transfer their attention from the very simplest organisms--bacteria and their viruses--to far more complex organisms such as fruitflies, mice, and human cells. The point of modern genetics is that these are no longer the "black boxes" of classical genetics, in which the nature of the genes was inferred from analysis of individuals derived from breeding experiments, and from microscopic examination of chromosomes. Instead, an organism such as a mouse can be considered as amenable to analysis on all levels, much as a play or symphony can be studied as an aggregate of words or notes (i.e., chemistry) or as an interacting system with deeper levels of meaning (i.e., physiology, development, evolution, normal and abnormal functions).

B. Mitochondrial and Chloroplast DNA: Maternal Inheritance and the Synthesis of Cellular and Population Genetics

1. The Nature of Human Mitochondrial DNA - A Paragon of Economy

GM 11726 (Attardi) California Institute of Technology

Each human cell contains several hundred mitochondria. These bacteria-like organelles are located in the cytoplasm and are the cell's power plants, converting the energy contained in foodstuffs into usable ATP energy. The mitochondrion is unique among cellular organelles in that it contains its own DNA and the molecular machinery to transcribe the DNA into RNA and translate the RNA into protein. The latter process is accomplished by structures called ribosomes, which are different in the mitochondria from those in the cytoplasm. Among the various differences, mitochondrial ribosomes are sensitive to the bacterial inhibitor chloramphenicol (CAP), while cytoplasmic ribosomes are not.

Each mitochondrion contains multiple copies of the mitochondrial DNA (mtDNA), and thus there may be thousands of mtDNA's per cell. The mtDNA is a closed circular molecule, 16,569 nucleotides long. The entire molecule has been sequenced and found to code for the two ribosomal RNA (rRNA) genes necessary for ribosome assembly, a set of transfer RNA's (tRNA) for alignment of the amino acids with the RNA coding sequence, and 13 to 14 genes specifying proteins. Five of these proteins have been found to be involved in the production of the energy storage molecule, ATP, but the function of the

remaining genes is unknown. However, the majority of the protein components of the mitochondrial apparatus, as well as the proteins involved in the replication of mtDNA and transcription of this DNA into RNA, and most of the proteins of the inner mitochondrial membrane, are encoded in nuclear genes and are synthesized in the cytoplasm and then imported into the mitochondria. Thus, the nuclear and mitochondrial genomes within each cell cooperate in the assembly of the mitochondrial protein synthesizing apparatus and of the enzyme complexes of the inner mitochondrial membrane.

In the past two years, the determination of the complete human mtDNA sequence in Dr. Frederick Sanger's laboratory in Cambridge, England, and the parallel studies carried out in the laboratory of Dr. Giuseppe Attardi at the California Institute of Technology on the synthesis, structure and map positions of the mitochondrial RNA transcripts in human cells have revealed the extraordinary features of gene organization and expression of the human mitochondrial genome. Two general approaches have been employed in the study of the genes in human mtDNA. The first has involved searching the newly revealed mtDNA sequence, using sophisticated computer algorithms, for stretches of nucleotides having coding capacity for proteins or which are capable of folding into the cloverleaf configuration characteristic of transfer RNAs. Supported by grant GM 11726-18, Dr. Giuseppe Attardi has followed a second approach. He located by RNA-DNA hybridization experiments (binding known RNAs to fragments of mtDNA) the DNA sequences complementary to functionally or structurally identifiable gene transcripts from Hela cell (a human cervical tumor cell line) mitochondria. He has located on the mtDNA the genes coding for the two ribosomal RNA species, the transfer RNA species, and the messenger RNA species which code for proteins. The two approaches have yielded perfectly matching pictures.

Dr. Attardi has shown that most of the mitochondrial genes, including the genes coding for the two ribosomal RNA species (12S and 16S RNA), fourteen transfer RNA species and twelve proteins are located on the heavy (H) strand of mitochondrial DNA, so designated on the basis of its density. The remaining genes, i.e., several transfer RNA genes and one protein coding gene, are located on the light (L) strand (Fig. 1). The arrangement of the genes on the H-strand is extremely compact: these genes are in fact immediately juxtaposed to each other, with no or only a few intervening, non-coding nucleotides. Correspondingly, the gene transcripts (the RNA copies of the genes), when aligned with the DNA sequence, form a continuum, without any intervening, non-coding sequences, extending over most of the H-strand. Never before has such an economy in gene organization been observed. Another striking feature is the arrangement of the transfer RNA genes, which separate the ribosomal RNA genes and the protein coding genes from each other with nearly absolute regularity (Fig. 1).

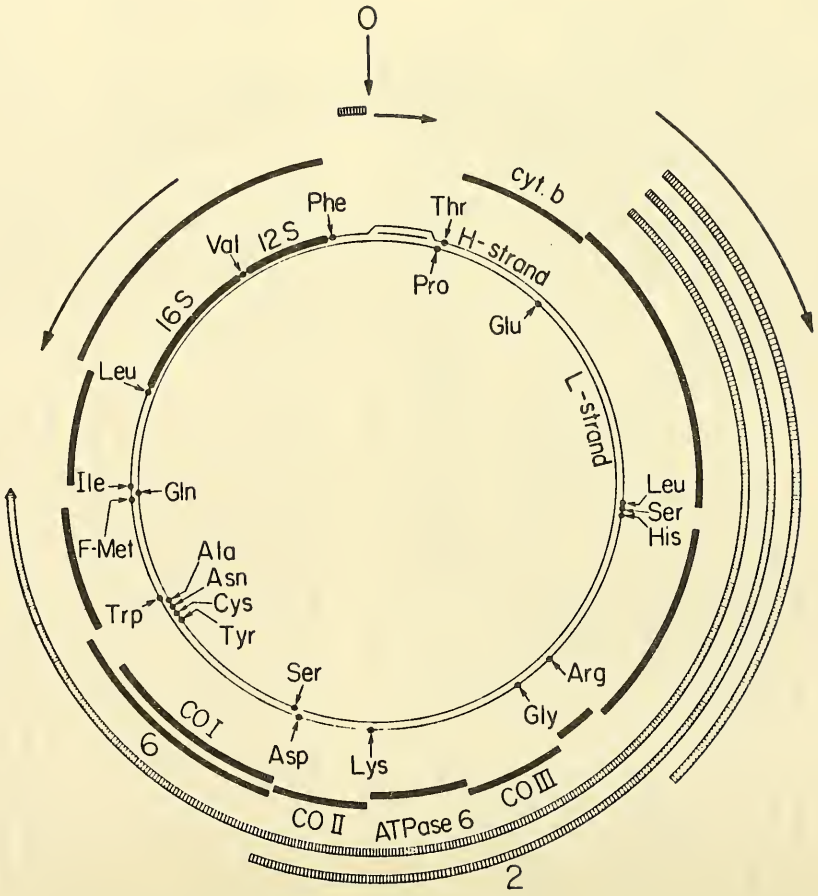

O
cyt. b
Phe
Thr
H-strand
Val
12 S
Pro
16 S
Glu
Leu
L-strand
Ile
Gln
Leu
Ser
His
F-Met
Ala
Asn
Cys
Tyr
Trp
Ser
Arg
Gly
COI
6
Asp
Lys
COIII
COII
ATPase 6
2

Figure 1

On the basis of the mtDNA map described above, Dr. Attardi has proposed a model in which the H-strand is transcribed in the form of a single molecule, which is processed by precise cleavages at the beginning and end of each transfer RNA sequence to yield, in most cases, mature RNA products. In this process, the secondary structure of the transfer RNA sequences may represent the main recognition signal for the processing (cleaving) enzyme(s), providing the punctuation in the reading of mtDNA information. A striking observation is that both the messenger and the ribosomal RNA species derived from the cleavage of the long precursor appear with the poly(A) tail at their 3'-end, a structural feature previously observed only in messenger RNA species. This observation strongly suggests that addition of the poly(A) sequence after transcription is linked in some way to the cleavage step releasing the 3'-end of the ribosomal or messenger RNA species from the flanking transfer RNA sequence, and is independent of the functional role of the RNA products.

The evolution to simplicity and economy which has marked the emergence of the human and, in general, the mammalian mitochondrial genome is apparent not only in its extraordinarily compact gene organization, but also in the structural features of the individual genes and gene products. Thus, the two ribosomal RNA species of human mitochondria are smaller than any known bacterial or cytoplasmic ribosomal RNA species. The transfer RNA species are likewise smaller, and many among them have a greater range of code word recognition than their cytoplasmic or bacterial counterparts; this accounts for the observation that the mitochondrial protein synthesizing apparatus can function with a smaller number of transfer RNA species than the cytoplasmic or bacterial machinery. The messenger RNAs lack, upstream of their coding sequence, a significant non-coding stretch. Such a leader sequence, found in all other known messenger RNAs, plays a role in ribosome attachment. Similarly, most of the mitochondrial messenger RNAs lack a complete termination signal for protein synthesis, i.e., the UAG or UAA code word, terminating instead with U or UA. In these messenger RNAs, the above-mentioned addition of the poly(A) tail at the site of cleavage of the long RNA precursor creates the termination signal by transforming U or UA into UAA.

Not only have the organization and structural features of the genes in human mitochondrial DNA been simplified in the course of evolution to an as yet unsurpassed degree, but also their mode and control of expression have evolved towards the simplest solutions. Thus, there is a single initiation site for transcription of the whole H-strand and one for transcription of the L-strand, both near the origin of replication. This provides, at the level of transcription, a coordinate control of expression of the whole genome. The necessary differential expression of the individual genes, in particular of the ribosomal RNA genes relative to the protein coding genes, is achieved through differences in the stability of the various RNAs. In addition, a frequent premature termination of H-strand transcripts past the ribosomal genes, which are located at the beginning of the transcription unit (Fig. 1), plays an important role in producing the large excess of ribosomal RNAs over messenger RNAs, which is required for the normal expression of the mitochondrial genome.

Dr. Attardi's laboratory has also been able to identify the specific proteins encoded by five of the human mitochondrial protein coding genes. These are the subunits I, II and III of cytochrome c oxidase (COI, COII and COIII, Fig 1), cytochrome b (cyt. b, Fig. 1), and a subunit of mitochondrial ATPase (ATPase 6, Fig. 1). These are all components of the oxidative phosphorylation apparatus which is responsible for energy production linked to respiration in all respiring organisms. A comparison of the sequence of these proteins with that of the homologous proteins in yeast has revealed a surprising degree of conservation. The preservation of the essential genetic function of mitochondrial DNA in organisms so far apart as yeast and man has to be contrasted with the extraordinary diversity in structure, gene organization and mechanisms of expression of the mitochondrial genome in different eukaryotic cells. Thus, in contrast to the compact and lean organization of human mitochondrial DNA, with its continuous genes mostly butt-jointed to each other and a nearly complete absence of non-coding stretches, yeast mitochondrial DNA has a very loose organization, with its genes being separated by spacers and several genes being discontinous. Similarly, the clustered arrangement of the transfer RNA genes in yeast mitochondrial DNA contrasts sharply with their scattered distribution in human mitochondrial DNA, where they separate the ribosomal RNA genes and the protein coding genes and have acquired the function of signals for the processing of the RNA. This is another striking example of the multiplicity of solutions to the same problem which nature has adopted in different organisms.

2. The Uses of Mitochondrial DNA Polymorphisms in Population Genetics
GM 24285 (Wallace) Stanford University; GM 22333 (Simpson) State University of New York, Stonybrook

Drs. Douglas Wallace (GM 24285) and Melvin Simpson (GM 22333) are among many geneticists who are discovering new ways of answering population genetics questions using mitochondrial DNA (mtDNA). This new approach is made possible by the discovery that mtDNA is inherited very differently from the DNA on chromosomes and because of a new synthesis of population genetics and cellular genetics techniques.

Mitochondria are organelles in the cell cytoplasm and are passed from mother to offspring through the egg. Fathers apparently contribute no mitochondria to the offspring. Thus, an individual and his sibs all have the same mtDNA sequence--that of their mother. Maternal inheritance of this sort means that the descendants of a female form a clone or a population of identical individuals.

By contrast, chromosomal genes are reshuffled each generation through genetic recombination and mixing of the genes from two parents. Each offspring gets one-half of its genes from each parent; conversely, each parent passes one-half of its genes to each offspring. Each generation, the correspondence between the original female parent and her descendants is reduced by one-half. This means that the descendants may not resemble the original parent. Therefore, tracing lineages through

many generations is a formidable task. Population geneticists have employed a variety of mathematical and statistical models to resolve the problems posed by the instability and fluidity of the chromosomal genome.

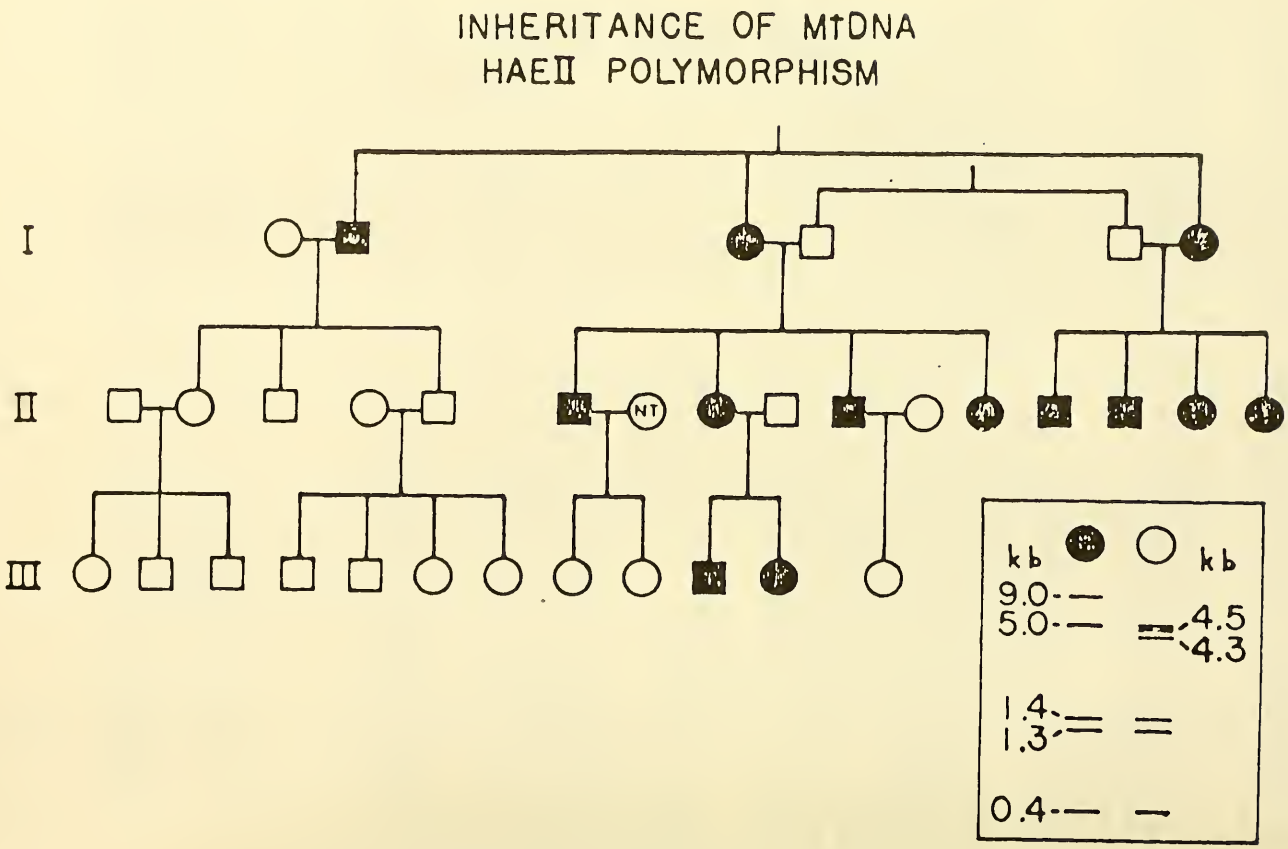
Mitochondrial DNA is much easier to study because it retains its integrity over many generations. Thus, studies of migration, evolution, and disease transmission, which are of much importance to genetics research but have been difficult to conduct, will be greatly simplified by using mtDNA.

Identification of mtDNA varieties

Most species are polymorphic for mtDNA; that is, DNAs with specific sequence variations can be found in the species. mtDNA varieties are easily identified by using restriction endonucleases to cut the mtDNA at particular sites where specific nucleotide sequences occur. The resulting fragments can be separated from each other on the basis of size by a variety of techniques. The restriction endonuclease Hind II breaks rat mtDNA into fragments that are 9000, 5000, or 2500 nucleotide bases long. If a mutation were to occur at one of the Hind II sites so that the enzyme could no longer attack and cut the DNA, only two fragments would be made from the new form. Thus, mtDNA polymorphisms are easily detectable and appear to preserve an accurate historical record of mutations.

Dr. Douglas Wallace has traced the existence of such mtDNA polymorphisms from specific ancestors throughout a large three-generation family. This family is shown diagramatically in Figure 2; females are represented by circles and males by squares. In generation I, three sibs inherited a "variant" mtDNA pattern (filled-in symbols). All children of the two females in generation I and both children of the married, variant female in generation II possess the variant mtDNA. All children and grandchildren of the variant male in generation I are "normal," as are the children of variant males in generation II. These data demonstrating clear maternal inheritance of mtRNA properties were obtained from blood platelet mtDNA. Dr. Wallace has obtained the same result using a different digesting enzyme and mtDNA isolated from fibroblasts from a smaller family obtained from the Human Genetic Mutant Cell Respository. These results represent the first demonstration of maternal inheritance of mtDNA in humans.

Figure 2


INHERITANCE OF MtDNA
HAEII POLYMORPHISM
I
II
III
NT
kb
kb
9.0
5.0
4.5
4.3
1.4
1.3
0.4

Study of genetic relatedness

By mapping the polymorphisms in isolated groups of a given species, population geneticists can measure how closely related populations are. The order in which mutations arose can often be determined and the order in which groups diverged from each other can be inferred.

Simpson (GM 22333) used six different endonucleases to study ten groups of Rattus rattus and fifteen groups of Rattus norvegicus, both common rat species. Simpson measured the degree of similarity or relatedness of their mtDNA's. The two species themselves shared about 84% of their mtDNA sequences. Populations within a species but from different geographical locations were closely related (90.4% - 99.8%). Populations separated by major geographical barriers, such as oceans or mountains, had more differences in mtDNA sequences. Because the number of differences in mtDNA sequences suggests the degree of genetic relatedness, these data are a valuable step in determining how species evolve. Two populations were especially interesting. Rats from Sri Lanka were very different from East Asian rats. Simpson hypothesized that these groups may really be subspecies on the verge of speciation. This view was borne out when crosses between East Asian and Sri Lankan rats produced only sterile offspring.

These results parallel findings on genetic divergence based on chromosome, enzyme, and DNA structure. However, the ease and accuracy of mtDNA technology is a great advantage in these studies.

Study of migration

Mitochondrial tDNA polymorphisms also allow researchers to study migration. A female who migrates to a new area produces a clone of offspring. As further migration occurs, clones will be established in new locations. By studying mtDNA polymorphisms in different geographic areas, it may be possible to retrace the history of migration of a species.

Dr. Douglas Wallace has begun studies of human migration to reveal the ancient history of mankind. Using the restriction endonclease HpaI to probe for changes in the mtDNA sequence GTTAAC, Dr. Wallace's group, in collaboration with Dr. Cavalli-Sforza, has already made a preliminary determination of the sequence of events leading to the origins of human differences. These studies indicate that man started in the Orient and radiated into Europe and Africa. More detailed studies are now in progress to confirm these preliminary observations and to extend and refine our knowledge of the record. A near perfect record of human biological history may thus have been found. Deciphering it could be one of the great anthropological achievements of our time.

3. Some Relationships Between Mitochondria and Bacteria: Sensitivity of Various Kinds of Ribosomes to Drugs
GM 24284 (Wallace) Stanford University

Ribosomes are nucleoprotein particles which are involved in the important process of protein synthesis. The cytoplasm of mammalian cells contains two kinds of ribosomes. One kind (mitochondrial ribosomes) is found inside the mitochondria, while the predominant species (cytoplasmic ribosomes) is found in the soluble portion of the cytoplasm (or, alternatively, is associated with the endoplasmic reticulum.)

A number of investigators have suggested that mitochondria are "living fossils" of ancient bacteria which infected the precursors of mammalian cells, established a symbiotic relationship, and subsequently became absolutely dependent on this cellular environment. The suggestion is supported, in part, by the finding that mitochondrial ribosomes are very similar to the ribosomes of many free-living bacteria, and are very different from cytoplasmic ribosomes. For example, mitochondrial ribosomes are generally sensitive to the antibiotic chloramphenicol (CAP) while cytoplasmic ribosomes are not sensitive to this drug.

Dr. Wallace has exploited the mtDNA sequence variations to probe the molecular basis of CAP sensitivity of the mitochondrial ribosome. The protocol first required generating CAP resistant human fibroblast cell lines and then finding out whether the mutations were generated in the nuclear or mitochondrial genomes. Cultured human cells were treated with mutagenic agents, and rare CAP resistant variants were isolated by growing the cells in medium containing CAP. To demonstrate that the mutations had occurred in the mtDNA, Dr. Wallace showed, as had others before, that transfer of mitochondria from such cells conferred resistance to recipient cells that had been originally CAP sensitive. The experiment was done by physically removing the nuclei of resistant cells and fusing the residual cytoplasm, containing the mitochondria, with sensitive cells. The resulting hybrids were found to be CAP resistant. The mtDNA association of the CAP resistance could further be demonstrated by correlating transfer of resistance with transfer of the specific cleavage-enzyme-detectable mtDNA sequence variations.

After ascertaining that the CAP resistance mutations were in the mtDNA, Dr. Wallace proceeded to determine the exact nucleotide changes responsible for this trait. One of the human mutants, resistant to high levels of CAP, was found to have a nucleotide sequence change in a region of the rRNA that is completely conserved from bacterial rRNAs through yeast and mouse mtDNA to human mtDNA. Yeast CAP resistance is known to result from a single nucleotide change in this same region of the mtDNA. Another human mutant line, which has a lower resistance to CAP, also had a single nucleotide change in a highly conserved region.

However, this change was 53 nucleotides away from the other mammalian mutations Dr. Wallace had detected or from the yeast mutation. This anomaly was resolved when the three-dimensional structure resulting from

pairing of rRNA bases was considered. The folding brings the two mutations to within 12 nucleotides of each other and demonstrates that they participate in the same active site. Interestingly, the sequence of the corresponding region in the nuclear-coded rRNA gene for the normally CAP resistant cytoplasmic ribosomes has a markedly different sequence in this region.

These results are the first demonstration of the site of mtDNA CAP resistance in mammalian cells, and demonstrate that bacterial and mammalian CAP sensitivity of mitochondrial ribosomes can be attributed to a common rRNA sequence. They also support the hypothesis that mammalian mitochondria are of bacterial origin.

The work of Drs. Attardi, Wallace, and Simpson demonstrates well the various kinds of research to which human mitochondria, and human mitrochondrial DNA, lend themselves. Attardi's work demonstrates the relationships between an unusually compact genome and its corresponding RNA and protein products. Other work described above shows how some features of maternal (cytoplasmic) inheritance in man can be employed to study problems as diverse as drug action and the genetic relationships between various human and rodent populations, and as an aid to historical research.

All of this work has relied on both technology and concepts developed by other NIGMS grantees in the past few years. It includes the use of restriction enzymes with highly defined specificities to characterize complex DNA molecules through their mode of fragmentation, and the ability to sequence long stretches of DNA efficiently and accurately.

4. A Mechanism for Maternal Inheritance of Algal Chloroplasts: Paternal Chloroplast DNA is Degraded
GM 22874 (Sager) Sidney Farber Cancer Institute

Mammalian mitochondria, and mitochondrial DNA, are maternally inherited simply because the egg's cytoplasm contains hundreds of mitrochondria. By contrast, the single mitochondrion of mammalian sperm, located in the acrosome, between head and tail, does not penetrate into the interior of the egg during fertilization.

However, this simple situation does not apply to the DNA of chloroplasts of the unicellular green alga Chlamydomonas. Understanding the mode of inheritance of this organelle, where photosynthesis takes place, has long been a challenge to molecular biologists.

Chlamydomonas cells differentiate into haploid gametes of two sexes. Cell fusion between gametes of the two different sexes produces a heterokaryon, composed of two nuclei which later fuse, and a common cytoplasm. Although nuclear inheritance is biparental, chloroplast genes are inherited from the female parent, but not from the males. Somehow, the male chloroplasts, and chloroplast DNA, which were present in the heterokaryon, disappear from the progeny of this fusion.

Dr. Sager has proposed that chloroplast heredity is regulated by methylation and restriction, and documents the hypothesis with her studies of the differentiation of vegetative Chlamydomonas cells into gametes. She has shown that the chloroplast DNA of vegetative cells is not methylated, but that within the 24-hour period during which vegetative cells become gametes, a rapid methylation of chloroplast DNA occurs in female, but not in male cells. Within a few hours after pairs of the cells fuse to form zygotes, there is total destruction of the chloroplast DNA from the male parent, as well as further methylation of the surviving chloroplast DNA which comes from the female parent. Dr. Sager has demonstrated methylation by two sensitive methods: 1) with the use of a pair of restriction enzymes--MspI, which cleaves DNA at the CCGG site whether it is methylated or not, and HpaII, which cleaves only nonmethylated CCGG; and 2) with a novel method of labeling every methyl cytosine base with antibody. Dr. Sager has concluded that DNA methylation regulates the maternal inheritance of chloroplast DNA. Evidence is accumulating to suggest that the same process may also govern maternal inheritance of chloroplast DNA in higher plants and of mitochondrial DNA in animals.

Dr. Sager is pursuing these studies on the role of methylation in gene expression in Chlamydomonas by examining the enzymes responsible for the methylation reactions. She has found that an enzyme of 60,000 molecular weight that exhibits a low level of methylation activity is found in vegetative cells. However, in female gametes and in zygotes, where methylation is very active, a new enzyme of molecular weight approximately 200,000 appears. Dr. Sager believes that the lower molecular weight form, upon a signal, aggregates into a multimeric active state. These results, both on the regulation of gene expression by methylation and on the regulation of methylation by enzyme aggregation, may add significantly to understanding the controls on cell differentiation and metabolism.

Dr. Ruth Sager's recent work on the significance of DNA methylation in regulating the genetics of a higher organism, Chlamydomonas, are based on work over the past decade, and parallel the work of investigators such as Salvador Luria and Werner Arber on the relationship of certain bacterial viruses and their hosts. It turns out here, also, that methylation of host DNA and the concomitant bacterial synthesis of enzymes to break down unmethylated viral DNA becomes a protective device to prevent a takeover of the bacterial cell by the virus.

It now appears that "selective silencing of DNA" via methylation occurs in a variety of important biological systems. DNA modified in this way may be protected from inactivation, or, in other situations, be blocked as a template for RNA and protein synthesis. Methylation may be the underlying reaction in effects as diverse as haploidization in barley and X-chromosome inactivation in female mammals. Dr. Sager's work is only one example of work supported proposing to examine the role of site-specific DNA methylation in cellular differentiation, development and, perhaps, disease causation.

C. Site-Specific Recombination, Transposons, and Human Restriction Fragment Length Polymorphisms: The Static Picture of DNA Replaced

1. Overview: Recent annual reports of this program (1979, 1980) have emphasized the growing significance of research on DNA sequences--transposons--that hop from one part of the chromosome to another, or even between chromosomes. It was becoming clear that such physical movement of DNA may play a significant role in processes as diverse as cellular differentiation, evolution or carcinogenesis. It was also becoming evident that there are close similarities between transposons and certain kinds of viruses--perhaps there are evolutionary relationships between the two!

Many laboratories, such as those of H. Echols, N. Cozzarelli, and J. Steitz, are now concentrating on the mechanisms by which transposons move from one site to another and on how their movement is controlled. Other laboratories, such as that of G. Fink, are studying transposition of genetic elements as events leading to mutation. Still other laboratories, such as that of Dr. L. Kedes, are studying solitary genes--orphons--similar or identical to genes that are found as clusters of many copies. There is speculation that such orphons may be a stage in the formation of new functional genes. Alternatively, other investigators lean toward the view that such DNA is a nonfunctional, fossil residue of functional DNA, i.e., "selfish DNA" or "junk DNA." The answers are not yet in as to which view, if either, will prevail.

The first human restriction fragment length polymorphism found among the Utah Mormons seems to be also the first transposon found in man. More recently, the group from Utah has reported that some other human polymorphisms that have been found are base-pair substitutions.

* * *

Some years ago, it was the fashion to think of DNA as a highly conserved, stable molecule. The analogy was to an "encyclopedia" of an individual's total genetic information, albeit with rare "typos," or mutations.

The emerging picture of DNA, highlighed by research on transposons, is one of a dynamic molecule, continually beset by physical (radiation), chemical (mutagens, carcinogens) and biological (viruses) insults from the environment, and actively re-edited by repair enzymes in a valiant and generally successful effort to preserve the original message. There are orderly, temporal processes, such as methylation of portions of DNA, that turn on the expression of some genes, and turn off others. In extreme cases (see section B4, for example) such reactions determine the protection or destruction of DNA. The transposition of DNA sequences between DNA molecules, or between chromosomes, corresponds, in this picture, to snipping out paragraphs from this once sacrosanct "encyclopedia" and inserting them elsewhere, where, read out of context, they may lose much of their original meaning or gain a new one.

The static picture of DNA has clearly been supplanted by one in which all parts are in constant motion. Our surprise, now, is the degree of genetic stability which appears to be maintained in the midst of constant change and transformation.

2. Mechanism of Site-Specific Recombination and Transposition
GM 17078-12 (Echols) University of California, Berkeley
GM 22729-06 (Cozzarelli) University of Chicago
GM 22729-10 (Steitz) Yale University
GM 28470-01 (Grindley) Yale University

It has recently become apparent that the movement of transposons has some similarity to site-specific recombination as exemplified by the integration of the bacterial virus λ into the E. coli chromosome. Integration of λ into the host chromosome has long served as the model system for prokaryotic site-specific recombination and transposition. Recent studies have yielded much new information concerning the biochemical mechanism and regulation of this process. The methods used in the analysis of λ integration are now being applied to the study of prokaryotic transposable elements and should yield rapid progress in our understanding of how these elements move about by a mechanism that involves DNA replication combined with localized recombination. Two key proteins involved in the reaction have now been isolated. This section of the report describes some of the features known about site-specific recombination and transposition and compares and contrasts these two specialized recombination mechanisms.

In 1962 Campbell proposed that λ DNA associates with the host chromosome by a recombinational mechanism. Subsequently genetic and physiological studies demonstrated that the Campbell model was correct and that the integration event involved a localized genetic exchange. By 1970 the enzyme requirements for the integration-excision reaction were defined: the forward insertion reaction requires the product of the phage int gene (Int protein), and the reverse excision reaction requires the products of the viral int and xis genes (Fig. 3).

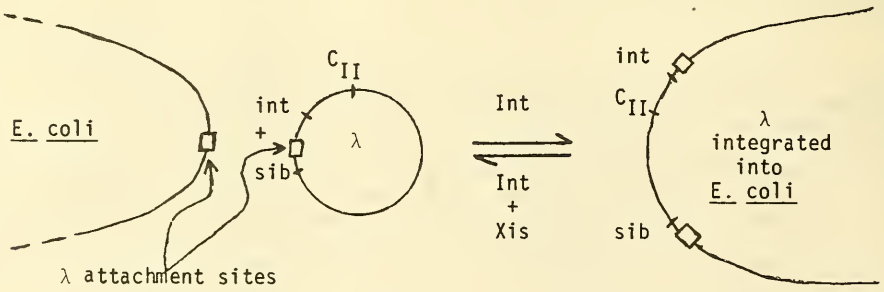


FIGURE 3

Experiments in the last ten years have focused principally on two areas: efforts to understand the regulation of the direction of the integration-excision reaction, and biochemical studies of the reaction itself. Notable progress has occurred in both areas.

In the biochemical area, Howard Nash, Arthur Landy, Echols and coworkers have purified the Int protein and demonstrated that Int protein binds tightly and specifically to the site on DNA where site-specific recombination takes place. The interaction may involve as many as four separate binding sites, extending over at least 200 bases in the DNA. While the recombinational exchange occurs within a 15-base "core sequence," the highest affinity site for Int protein is centered some 75 bases away. Thus the role of Int clearly involves specificity, but the reaction mechanism must be extremely complex. Int protein has DNA unwinding (topoisomerase) activity and therefore may also participate directly in DNA strand exchange.

Bacteriophage λ regulates the integration-excision reaction very precisely because it is a critical part of the control of development along one of the two viral pathways, lysogenic versus lytic. The lysogenic response requires integration of the viral DNA, while the lytic response requires that viral DNA should not be integrated. In the case of an integrated virus induced to lytic growth, excision of the viral DNA is required.

The choice between integration and excision is based on the different catalytic requirements for the two reactions. As shown in Fig. 3, the only phage-specified protein required for integration is Int, whereas excision requires the viral Int and Xis proteins. Thus increased synthesis of Int with respect to Xis will favor integration. The Campbell and Echols groups have shown that the phage cII protein, which acts by turning on *int*, is responsible for the major part of this regulation. In addition, Guarneros, Echols and coworkers have shown that the viral gene *sib* depresses *int* gene expression. *Sib* presumably prevents unwanted integration in a cell destined for the lytic response. The fact that the *sib* gene is physically separated from the *int* gene by the recombination reaction that inserts the viral DNA, probably accounts for the fact that the repression of *int* by *sib* does not occur when an inserted virus is induced to lytic growth (see Fig. 4). In this situation Int is needed together with Xis, to excise the virus from the bacterial chromosome. The ability of both *CII* and *sib* to regulate the *int* gene is a fascinating example of multiple control mechanisms providing precise physiological regulation of complex cellular reactions.

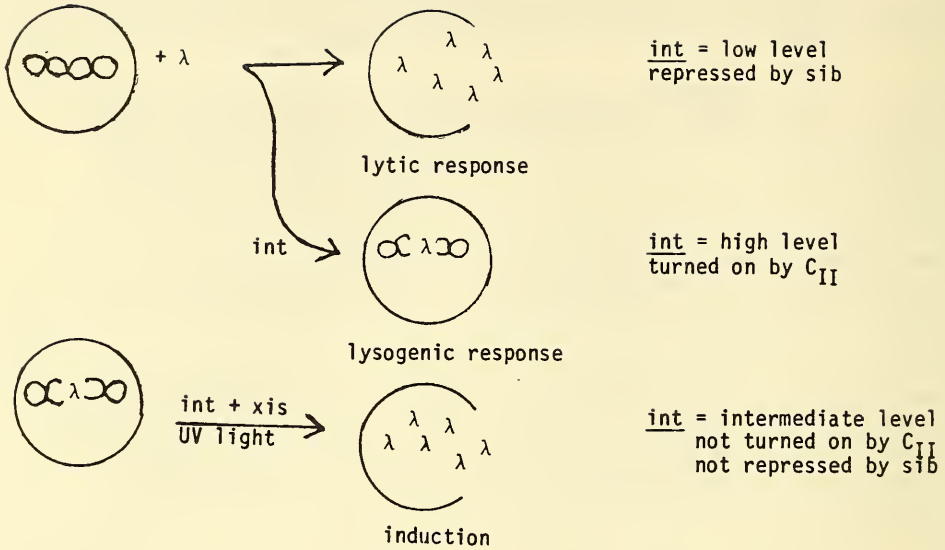


FIGURE 4

Phage λ can be viewed as a highly specialized transposon. Recently it has been shown that a site-specific recombination reaction very similar to that of λ forms part of the transposition mechanism of some transposable elements, e.g., Tn3 and γδ. When these transposable elements transpose from one chromosome to another, the two chromosomes first fuse to form one large chromosome containing two copies of the transposable element. This reaction is catalyzed by the product of gene tnp A, called transposase, and must involve some DNA replication, since the transposable element is duplicated in the process. A site-specific recombination then takes place between the two copies of the transposable element, yielding two chromosomes each with a copy of the transposable element. This reaction is catalyzed by the product of gene tnp R, called resolvase. Both tnpA and tnpR are located on the transposable elements. The two reactions are shown in Fig. 5 on next page.

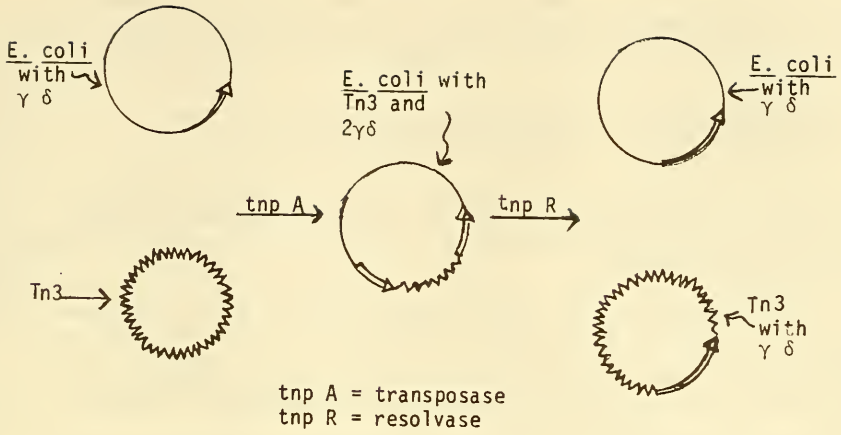


FIGURE 5

Cozzarelli and Steitz, working independently, have been able to isolate the two transposon-coded enzymes involved in transposition of Tn3 and γδ transposons. Cozzarelli has isolated the transposase, the enzyme that is required for the first reaction shown in the diagram. Various host proteins are also required, of course, but these presumably are proteins that have a normal function in other cell processes. Although transposase has been shown to bind tightly to single-stranded DNA, its enzymatic activity in vitro has yet to be discovered.

Steitz and coworkers and Grindley have been able to demonstrate the resolvase component of the transposition reaction in the test tube. (This corresponds to the second reaction in Fig. 5. They have constructed a plasmid containing the two transposons γδ and Tn3. In the presence of purified resolvase this plasmid will break down in vitro into two component parts, each carrying one transposon. The reaction takes place in a region of DNA that is homologous between the two transposons and is 18 base pairs long. Only super-coiled DNA containing two of the 18 base pair sites, Mg^{++} and a suitable buffer are required. The mechanism of the reaction appears to involve a double-stranded cut by resolvase at each of the 18 base-pair sites, together with covalent attachment of resolvase to the newly formed 5' ends of the DNA.

There are some obvious similarities between integration-excision of λ and the reaction catalyzed by resolvase. In both cases the reaction is site-specific and reciprocal; it occurs within a short region of homology but is independent of ordinary homologous recombination; it requires the product of a gene carried by the element; both reactions occur in the absence of DNA synthesis or an energy source. There are also differences between the two systems. λ recombination requires host as well as viral factors, while the resolvase reaction requires only that protein; λ recombination occurs efficiently both intramolecularly and

intermolecularly, while resolvase works efficiently only as an intramolecular reaction between directly repeated sites. Further studies will undoubtedly clarify the nature of these reactions and how they are controlled in the cell.

3. Gene Instability in Yeast Caused by Transposons
GM 15408-14 (Fink) Cornell University

Dr. Gerald Fink has reported the first rigorous demonstration of transposition in eukaryotes by studying an unstable mutation in the his4 gene of yeast. (This section represents an update of a highlight mentioned in the 1980 annual report.)

Unstable mutations in bacteria have been shown to be produced by transposition of movable pieces of DNA (transposons) to a location close to or within a gene, which is thereby inactivated. The instability arises from the fact that the transposon will move out of the gene at a high frequency, often leaving behind an intact, functional gene.

Bacterial transposons share certain features. They have terminal repeated sequences that are required for transposition. While transposition is usually conservative--that is, it results in a copy of the transposon at both the new and the old location--occasionally a transposon is lost from a particular location, and concomitantly a number of chromosomal aberrations, including deletions, translocations, transpositions and inversions, can occur. At least two transposons are known to carry genes which make the enzymes necessary for transposition. Another remarkable feature of all bacterial transpons is that, upon integration, they generate a short oligonucleotide duplication of the target DNA at either end of the transposable element.

Dr. Fink has been studying two unstable mutants of his4, called his4-912 and his4-917. Both are spontaneous mutations. Revertants appear at high frequencies and, in the case of his4-912, carry chromosomal aberrations in the vicinity of his4. The two mutations have each resulted from insertion of an element about 6000 base pairs long; however, the elements in the two mutants differ from each other in nucleotide sequence. A more detailed study of their structure revealed that the his4-912 insertion element is very similar to Ty1, a family of repeated yeast DNA sequences. His4-917 also has homology to Ty1 in about one third of its length, but the rest of this element is related to other repetitive DNA in yeast. Both elements have a 300 base pair repeat at each end.

When the genes into which the his4-912 or -917 elements have inserted are examined, it is found that the transposons have inserted by a mechanism very similar to that in bacteria. A 5 base pair duplication of his4 DNA has occurred at the site of insertion, and the insertion represents one complete copy of the element only, with no additional DNA involved. Both elements appear to prefer a region of the gene that is rich in AT sequences, a base sequence preference shared by Ty1 elements.

Although this is the only case in eukaryotes where a detailed study has demonstrated a transposable element with all the characteristics of bacterial transposons, there are many examples of presumed eukaryotic transposons. These include at least some of the repeated gene families of drosophila (412, copia, and 297) and the RNA tumor viruses. A fascinating aspect of the his4 transposable elements is their regulation by the cell. When these elements insert into the his4 gene, the gene comes under control of unlinked regulatory elements known as spm1, spm2, and spm3. These regulatory elements can supress the mutant effect of the transposons in some cases and the frequency of reversion of the mutation, i.e., the frequency of transposition, in others. The whole system therefore is beginning to look very much like the controlling elements of maize discovered in the 1940's by Barbara McClintock. Although her work represents the first discovery of transposons, their true nature becomes apparent only through the use of modern genetic techniques.

4. Orphons are Genes That Have Lost Their Families
GM 17995-09 (Kedes) Stanford University

A class of newly discovered genes was recently reported by a group in the laboratory of Larry Kedes (Childs, et al., Cell 23: 651, 1981). These genes, which have been named orphons, are solitary genes derived from large clusters of multigene families and located at some unknown distance from their families. The existence of orphons has exciting implications for the evolution of genetic information.

Orphons related to the H3 early histone gene of sea urchins were the first to be detected. There are five different genes which code for the histone proteins synthesized in sea urchin embryos. The repeated sequence of these genes (H1-H4-H2B-H3-H2A) occurs in a cluster of hundreds of copies. Kedes' group knew that the region of DNA containing the histone genes does not contain nucleotide sequences which are sensitive to enzymatic cleavage by the restriction endonuclease Bam H1. When the total DNA was treated with Bam H1, therefore, the histone coding region remained as a large DNA fragment which was separated from smaller DNA fragments by electrophoresis on agarose gels. The separated fragments were then incubated with a cloned histone H3 gene which had been radioactively labeled and which can hybridize (bind) with complementary strands of H3 DNA. As expected, the radioactive probe hybridized with the large fragments of histone coding DNA. In addition, however, the H3 gene probe hybridized with several smaller pieces of DNA, suggesting that these fragments contained variant genes (orphons) for H3 which are not part of the large histone cluster. Interestingly, the orphons in each individual sea urchin are found in different Bam H1-digested fragments, implying that their locations on chromosomes may be variable.

Orphons related to each of the other early histone genes of sea urchins have also been detected and range in number from a few to more than 20 in an individual. Orphons related to histones in Drosophila and orphons related to the tandemly arranged ribosomal genes in yeast and Drosophila have also been detected, leading to speculations on the possible origin and evolutionary significance of these genetic elements.

What is a polymorphism? If, in any population of people, we had, in addition to an (arbitrarily selected) "normal gene," a variant allele that has an appreciable frequency, we would call this a polymorphism. The most informative polymorphisms in human genetics are those with a frequency above about 10%. Up to now, the usefulness of any human gene mapping strategy employing polymorphic markers (i.e., ABO and Rh blood groups, hemoglobin variants, carbonic anhydrase, alpha-1 antitrypsin, etc.) has been limited by the paucity of such loci and also, often, by their low level of polymorphism. After decades of study of blood cell antigens and blood protein polymorphisms, only about twenty have been found that can be considered useful for linkage studies, and this is not enough, by far.

Restriction fragment length polymorphisms are detected as differences in length of DNA fragments, after digestion with one of the many restriction enzymes (nucleases that cut DNA at very specific sequences, and that thus produce long fragments), electrophoresis on agarose gels (which separates the fragments by size), and hybridization with one of a number of radioactive probes (which determine which of a multitude of sites, and therefore which polymorphism, is actually "seen" by the investigator). Unlike antigens and enzymes, the same technology is used to study all RFLP's, only the probes are different.

About eight RFLP's have been found through studies of specific human genes, such as globin or insulin. However, the need for a genetic linkage map saturated with many loci, and the existence of the requisite technology, suggested to Botstein, Skolnick and their colleagues that a search for arbitrary DNA fragments, designed to maximize the efficiency of uncovering useful polymorphisms, was needed. In November 1980, the first such arbitrary polymorphic marker in human DNA was described by White and collaborators. The marker described by White has many of the characteristics associated with transposable genes (or "jumping genes") in such organisms as bacteria, yeast, and fruit flies. It is therefore possible that this new human marker represents the first transposon found in man.

Since that time, another 17 arbitrary RFLP's have been found, and thus these investigators are well on their way to developing a new tool for human genetic mapping. Most of the newer polymorphisms found were base-pair substitutions, detected by a loss or gain of restriction enzyme cleavage sites. By contrast, insertions, as would be produced by a transposon, increase the size of the fragment seen, regardless of the restriction enzyme used, as long as its fragments span the relevant region. However, it should be emphasized that the frequency of the various polymorphisms seen does not tell us much about the nature of mutations in human DNA. Like any human search procedure, the methods and the tools used determine what kinds of things are found! Enzymes that cut at many places, or at sequences particularly sensitive to mutation, will tend to "see" base-pair substitutions. Enzymes that cut out larger pieces from DNA will, by contrast, tend to "see" insertions, including transposons.

Drs. Skolnick and White in a recent paper state that "we suspect that a new era for mapping genetic diseases is in reach." Many people feel that their suspicion is well-founded.

When cells divide, homologous chromosomes pair up and may exchange segments of DNA by the process known as "crossing over." In regions of the DNA containing repeated sequences, there is greater potential for unequal crossing over because the homologous chromosomes can match up at many points. Misalignment produces a segment of DNA which is looped out and may be integrated in the genome at another location to form an orphon. If such a process occurs during gamete formation, the randomly integrated orphon becomes a stable part of the individual's genome.

A. Leder, S. Swan, F. Ruddle, P. D'Eustacio, and P. Leder recently reported that α-globin-like genes are dispersed on three different chromosomes in the mouse (Nature 293: 196, 1981), and although globin genes do not occur in tandemly arranged multigene clusters, these dispersed genes may represent another example of orphons. (This work was supported in part by a grant to Dr. Frank Ruddle, Yale University, GM 09966.)

It is not yet known whether orphons have special functions. However, orphons located in an enironment removed from their normal controls may not be subject to the correction mechanisms which keep members of the tandemly repeated genes homologous. Orphons might be free to diverge in nucleic acid sequence and become subject to new patterns of regulation or even code for new proteins. Continued investigation of orphons, and in particular their sequence divergence, could lead to a new understanding of gene evolution.

5. Human Restriction Fragment Length Polymorphisms (RFLP's): The "New Genetics"

GM 29789-01 (White) University of Utah
GM 27192-02 (Skolnick) University of Utah
GM 21253-06 (Botstein) Massachusetts Institute of Technology
GM 21891-07 (Davis) Stanford University

The advent of recombinant DNA technology has opened numerous horizons in human genetics. Among them is the possibility of developing a genetic linkage map of human genes, saturated with restriction fragments length polymorphisms (RFLP's.) As recently suggested by David Botstein and his colleagues, "pedigrees in which inherited traits are known to be segregating can than be analyzed, making possible the mapping of the gene(s) responsible for the trait with respect to the DNA marker loci, without requiring direct access to a specified gene's DNA. For inherited diseases mapped in this way, linked DNA marked loci could be used predictively for genetic counseling."

Geneticists estimate that 150 such markers would suffice for the disease linkage studies that they would like to do. These studies will be aided by the availability of computerized, well-documented pedigrees of the large Mormon population in Utah, a project supported in part by NIGMS. Many geneticists are enthusiastic about this new molecular approach to human genetics and, because of its potential, are referring to it as the "New Genetics."

D. A Model for the Secondary Structure of 16S Ribosomal RNA

GM 17129-10 (Noller) University of California
GM 14825, GM 19843, GM 27576 (Cantor) Columbia University
GM 07631 (Carbon) University of California
GM 21688 (Fox) University of Texas
GM 25023 (Held) Roswell Park Memorial Institute
GM 27201 (Hill) University of Montana
GM 24034, GM 27566 (Lake) University of California
GM 20427 (Nomura) University of Wisconsin
GM 20147 (Pace) National Jewish Hospital
GM 11738 (Santer) Haverford College
GM 25701 (Schaup) Oregon State University
GM 29265 (Schwartz) New York Medical College
GM 17924 (Traut) University of California
GM 22807 (Zimmerman) University of Massachusetts

A landmark in ribosome biology has occurred this year with the publication (Noller and Woese, Science 212; p. 403, April 24, 1981) of a structural model for a major RNA component of ribosomes. This model represents the synthesis of several types of approaches to the structure of ribosomal RNA as performed by several laboratories over a period of several years. The published model is based on many lines of evidence, is conservatively stated, and is utilized by the authors to propose functional and evolutionary hypotheses. As was noted by Noller and Woese, the work upon which the model is based was performed by a large number of investigators, most of whom have been supported by the National Institute of General Medical Sciences.

The E. coli ribosome has a molecular weight of 2.6×10^6, comprising about 52 different proteins as well as 5S, 16S, and 23S ribosomal RNA's (rRNA's). This ribosome, called the 70S ribosome, can be dissociated into 30S and 50S subunits, both of which contain RNA and protein in noncovalent association. The 30S subunit contains the 16S rRNA (1542 nucleotides) and a single copy of each of 21 different proteins. The 50S subunit contains the 5S rRNA (120 nucleotides), the 23S rRNA (2904 nucleotides), a single copy of each of 31 different proteins, and four copies of one protein. The model presented in this work is for the structure of the 16S rRNA (the only RNA) component of the 30S subunit. This 16S rRNA is highly interactive with proteins of the same (30S) subunit, and also with the 50S subunit.

In recent times certain attributes of ribosomes have become recognized. Ribosomes are complex, the constituents are highly interactive, and ribosomes from one species are surpisingly competent to function in other species. The complexity of ribosomes implies an extraordinarily high degree of asymmetry. At present, ribosome crystals suitable for x-ray diffraction analysis are not available; however, if or when such crystals become available, crystallographers are likely to be challenged with a formidable problem. In the absence of conventional physical approaches to the structure of the ribosome, various ingenious approaches have emerged. These approaches include studies concerned with determining the spatial location of the ribosomal proteins by chemical cross-linking

(Traut, GM 17924), by neutron diffraction, by singlet-singlet energy transfer (Cantor, GM 14825, GM 19843, and GM 25756), and by immunoelectron microscopy (Lake, GM 24034, GM 27566). The results of these studies appear to be converging on a mutually consistent set of coordinates which define the location of several proteins in the 30S ribosomal subunit.

The information which is most essential to developing a model for the secondary structure of a ribosomal RNA molecule is the primary sequence of the nucleotides and primary sequences of the corresponding molecules from other species ("comparative sequences"). Nucleotide sequences of the E. coli 16S and 23S RNA's are now known (Noller, GM 17129, and Carbon, GM 07621). The 16S rRNA sequence for another bacterium, Bacillus brevis, is nearly complete (Noller) and extensive sequence information on 16S rRNA's exists in the form of ribonuclease T1 oligonucleotide catalogs from more than 150 species of bacteria (Fox, GM 21688). Recently sequences for chloroplast 16S RNA, mitochondrial 12S and 16S RNA's (analogous to bacterial 16S and 23S rRNA, respectively) and part of a yeast 18S RNA (analogous to bacterial 16S RNA) have also become available. This sequence information has been augmented by the availability of several direct biochemical indicators of structure which will be discussed in the following sections.

Methods of Derivation of the Model

The nucleotide sequence of E. coli 16S rRNA was obtained by sequencing the corresponding DNA. A first consideration in proposing major aspects of the secondary structure of an RNA was the search for base-paired (anticipated to be largely helical) regions. Regions of the 16S rRNA molecule which were considered candidates for base-pairing were generated by computer program. Those sequences which would permit helices with four or more base pairs and with predicted stabilities of at least the order of those seen in the well-established structure for transfer RNA (tRNA) stems were provisionally accepted. The authors considered a high degree of commonality in sequences from different species to be the most significant evidence for secondary structure. Both tRNA and 5S RNA secondary structure models had been derived mainly from comparative evidence, as was the Watson-Crick model for base-pairing of DNA itself. In utilizing comparative sequence data, an underlying assumption was that the secondary structures of 16S rRNA from different prokaryotes would be very similar. This is supported by the finding that 16S rRNA from different bacteria can be interchanged in reconstitution experiments in vitro. Conservation of a base-paired region, in spite of some variation in paired sequence, was considered a convincing argument for a duplex structure in that region.

Evidence suggesting the existence of secondary (base-paired) structure was contrasted with evidence supporting absence of secondary structure at any given site. The ability to modify a region with chemicals that react only with single strand, the ability to obtain ribonucleoprotein fragments, or the existence of naked 16S rRNA fragments were taken as strong evidence of lack of secondary structure. The reliability of chemical modification as a test of secondary structure has been supported by studies with phenylalanyl-transfer RNA from yeast, where there is complete agreement

between the structure inferred from the reactivity of various sites to chemical modification and the structure obtained by x-ray crystallographic analysis. A potential danger in the chemical modification method of assignment is the possibility of shielding by tertiary structure or protein binding. These possibilities were assessed during later refinement of the model. Another method sometimes utilized to discern single-stranded regions, nuclease susceptibility, was considered to be less reliable because it is known that an initial cut may lead to some unfolding of the RNA. As 16S rRNA has a large number of target sites, use of this criterion was considered particularly suspect. Noller particularly exploited direct isolation of sets of RNA fragments, complexed with each other (thus requiring the respective fragments to contain complementary sequences), to confirm base-pairing.

The Model

The model for the secondary structure of 16S rRNA proposed by Noller and Woese is reproduced here (see Fig. 6). The general structure appears to be governed by sets of long-range interactions which partition the 16S rRNA into several domains. For example, a helix formed by the interaction of nucleotide residues 27-37 and 547-556 organizes residues 1-556 into a 5' terminal domain seen at the lower left of the diagram.

Assuming an average internucleotide distance of three angstroms (Å), the proposed 16S rRNA structure will fill an area 300 by 450 Å. The overall dimensions of the 30S ribosomal subunit, of which 16S rRNA is a component, are suggested to be 55 by 220 by 220 Å by lw -angle x-ray scattering (Hill, GM 27201) or 80 by 190 by 250 Å by electron microscopy (Lake). This model would then require twofold condensation to fit into the observed dimensions, a figure which is reasonable.

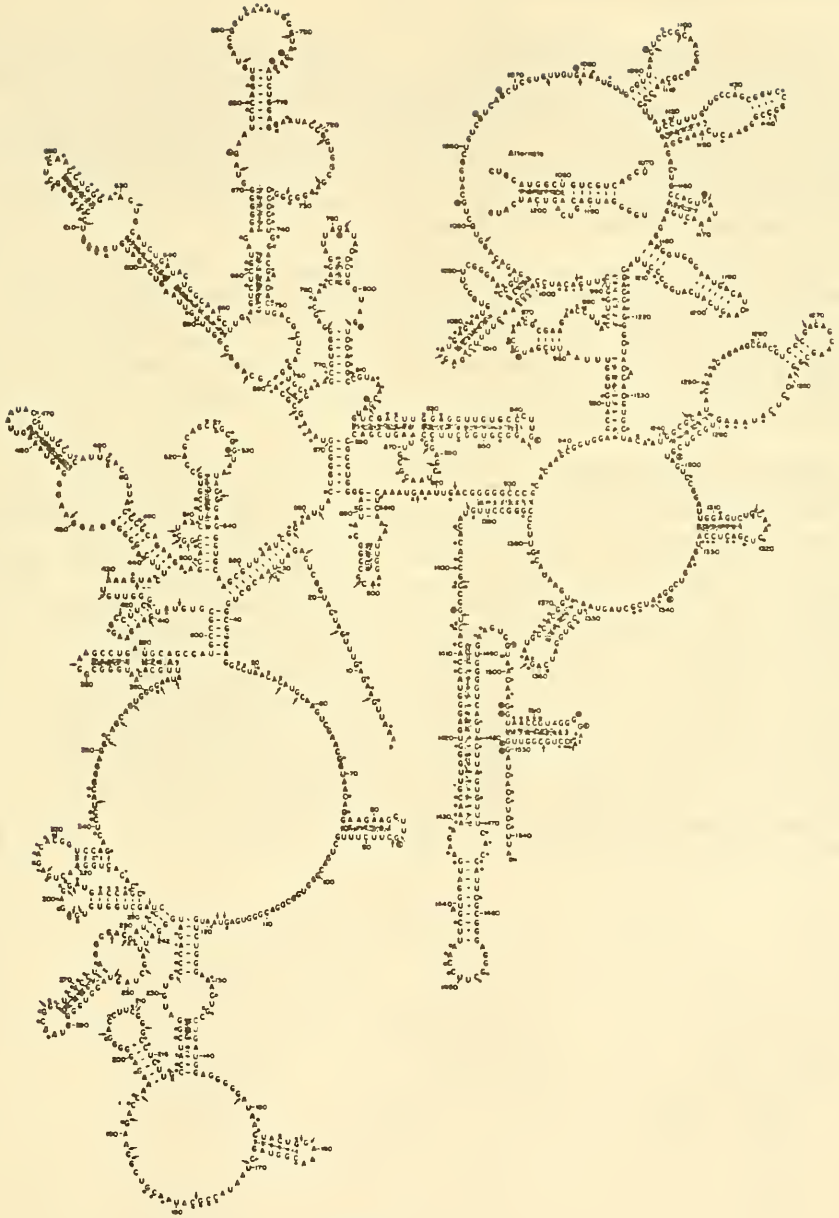


FIGURE 6

Functional Attributes of the Model

Patterns of Sequence Conservation. The rRNA genes are included among those genes whose sequence has least changed during evolution. The existence of such extensive conservation implies a strong functional constraint on rRNA's. Therefore, patterns of sequence conservation provide clues to function. A major feature of 16S rRNA is that strict sequence conservation tends to occur in nonhelical regions and in regions, moreover, which tend to be exposed in isolated rRNA. By analogy with tRNA, these regions may be important to higher level structure.

At the other extreme are regions which are supervariable, even within a species. These regions tend to occur in helices and to contain a high proportion of abnormal base pairs (U•G, even A•G), and some appear to bind protein. Drs. Noller and Woese suggest that this last attribute may be highly significant if proteins stabilize the helices, if mutations in the rRNA are more easily compensated due to protein binding, and if mutational drift and even evolution occur more easily at these sites.

Ribosomal Binding Sites. An aspect which has been of considerable interest to the community for some time is the location of specific regions on the RNA which recognize ribosomal proteins. This model assigns recognition regions on 16S RNA for three proteins. All three regions turn out to be irregular helical regions, contain G•U pairs or single-base bulges, and, interestingly, are phylogenetically variable. Noller isolated such protein binding sites ("structural domains"), which were readily isolated as complexed, intact structures. These regions fit proposed non-helical domains of the model excellently. For example, it has been known for some time that ribosomal protein S4 binds to the 5' region of the 16S rRNA molecule. The placement of this domain was supported by isolation of appropriate protein-RNA complexes. Electron microscopy of S4-16S rRNA complexes also matches the proposed domains. The S4 domain is also known to overlap the S20 protein-binding region, and the overall placement of these two proteins has been supported by UV-induced cross linking data. The placement of important protein-binding domains is presented in Fig. 7.

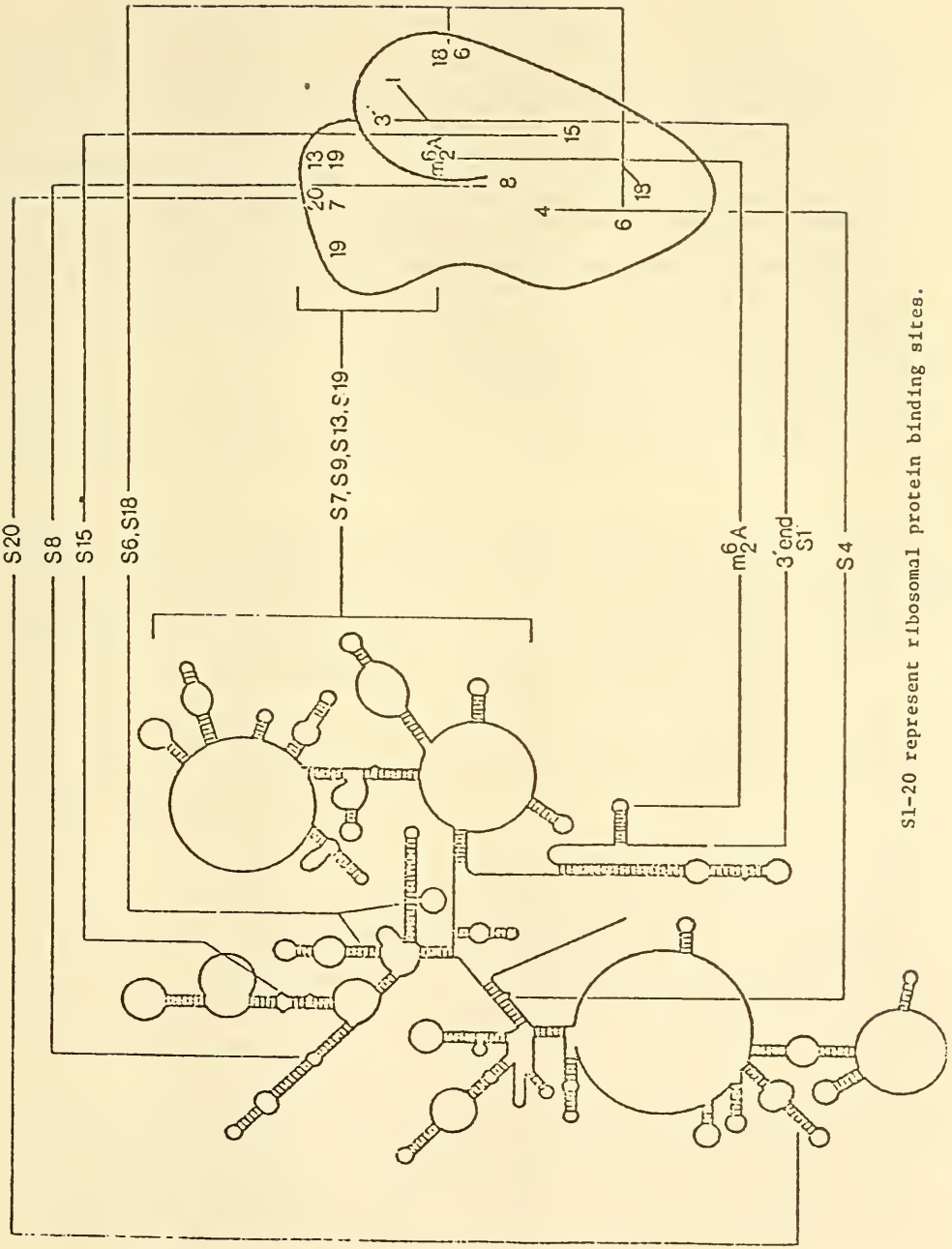


Figure 7. Relationship of 16S rRNA Secondary Structure to the Lake 30S Subunit Model

Figure 7 illustrates well that when molecular biologists deal with the structural aspects of ribosomes, they work in a region of overlap between very large molecules and very small organelles. The left portion of the diagram represents the chemist's view of what portions of his view of the 16S RNA molecule complex to certain ribosomal proteins. The right portion represents the morphologist's view of the nucleoprotein structure seen. The difficulty, of course is that, to the chemist, the molecule is very large, and its analysis is difficult and complex. To the morphologist, the structure is very small, and near the limits of resolution of the best instruments. We are working near the limits of both disciplines!

This model will set the stage to examine the next important questions in ribosome biology, those of tertiary structure and of precise interactions between the 16S ribosomal RNA and other proteins and RNA's. A similar model for the secondary structure of 23S rRNA has been completed by the same investigators and will be published soon.

SCOPE

This report reviews the activities of the Pharmacological Sciences Program for FY 1981.

OBJECTIVES

The aims of the Pharmacological Sciences Program are to provide an improved understanding of the biological phenomena and related chemical and molecular processes involved in the actions of therapeutic drugs and their metabolites as well as to effect the design and synthesis of new medicinal agents.

RESEARCH

Each year as we review the research highlights submitted by our grantees, certain themes present themselves. This year, several of the highlights, when examined together, point to the sometimes artificial boundary between pharmacology and endocrinology -- both fields in which the event of interest begins with the interaction of a molecule and a receptor.

One of the frustrations that has affected certain segments of the pharmacological research community has been the paucity, or inapplicability to pharmacological problems, of the simple bacterial or single cell systems which have provided models to the biochemists and cell biologists. This year's research highlights from grantees of the Pharmacological Sciences Program indicate the introduction of some novel systems which are contributing to an understanding of the interaction between molecule (drug) and receptor. Even the lowly E. coli are doing their part to unravel the stereochemistry of membrane receptor/drug interactions.

Again demonstrating the diverse ways in which scientific fields merge, investigations of natural products and biosynthetic mechanisms related to the shikimic acid pathway and the biosynthesis of vitamin K have been complemented by genetic studies. As described below, the genetic controls of the various parts of this pathway are currently being unraveled.

Work by a grantee of the Pharmacological Sciences Program has once again given an example of the ways in which support of basic research contributes to the solution of problems of significant environmental and economic concern. Dr. Barry Sharpless, supported in the Biorelated Chemistry portion of the Program, has developed new synthetic methodology ("asymmetric epoxidation") which has significantly simplified synthetic routes to complicated molecules having chiral centers. This methodology has been applied to synthesis of the gypsy moth pheromone (see below) and is expected to find even greater application in the syntheses of chiral vitamins and drugs.

Over the past few years, the National Institute of General Medical Sciences has been developing a series of pamphlets directed to high school and college science students and the public. Mrs. Maya Pines, a professional science writer, worked with the staff and grantees of the Program to develop "Medicines and You," the third volume in the series "A New Medical Science for the 21st Century." So far, over 20,000 copies have been requested from the Institute's information office, primarily for classroom use.

RESEARCH TRAINING

Since 1975 the Pharmacological Sciences Program has supported research training in the pharmacological sciences and clinical pharmacology under the authority of the National Research Service Act. Institutional fellowships provide for the support of multidisciplinary predoctoral training in the pharmacological sciences and postdoctoral training in clinical pharmacology. Individual fellowships provide for postdoctoral training both in pharmacological sciences and in clinical pharmacology.

The institutional predoctoral training programs in the pharmacological sciences are multidisciplinary in nature, providing students with opportunities to receive training not only in pharmacology but in medicinal chemistry, immunology, neuroscience, biochemistry, physiology and other related biological sciences as well. Despite the multidisciplinary nature of these programs, it is still the intent that all trainees supported in the area of pharmacological sciences receive a thorough grounding in pharmacological principles and their application to all aspects of biomedical research. In FY 1981, there were 43 institutional predoctoral training programs supported in 25 states. Additionally, 36 individual postdoctoral fellowships were also awarded in the area of pharmacological sciences.

The Pharmacological Sciences Program maintains a continuing commitment to clinical pharmacology by providing support through multiple mechanisms. Research Career Development Award applications are accepted by this Program only from those investigators who have expressed a commitment to research careers in clinical pharmacology. Both the Pharmacological Sciences Program and the National Institute of General Medical Sciences intend that this mechanism will give such individuals the opportunity to develop into outstanding independent investigators capable of applying basic pharmacological principles to problems in clinical medicine. One new award was made in FY 1981, and support for two other clinical investigators is continuing. Prior to July 1, 1977, Research Career Development Awards were made in diverse areas of the pharmacological sciences, and support has continued for one such individual. Institutional postdoctoral fellowship programs in clinical pharmacology are designed to train physicians in the application of pharmacological laboratory principles to clinical research. In FY 1981, there were 13 such programs in 11 states across the country. Individual postdoctoral fellowships are also available to qualified applicants in the area of clinical pharmacology, and in FY 1981, one such fellowship was awarded. In addition, the Pharmacology Research Associate Program (see below) provides an option for qualified and interested physicians to pursue an individualized curriculum in clinical pharmacology at the National Institutes of Health under the guidance of Dr. William Z. Potter. This is complemented by a two-semester seminar series in clinical pharmacology approved for continuing medical education credit.

The Pharmacology Research Associate Program of the National Institute of General Medical Sciences provides opportunities for 22 highly competitive postdoctoral fellows to spend two years pursuing their research interests in the intramural laboratories of the National Institutes of Health. There are currently 57 formal preceptors directing research in such areas as the molecular mechanisms operative in central and peripheral neurotransmitter systems, the mechanisms and modification of cellular control, and genetic and environmental influences on physiological

responses. In addition, fellows with a pharmacological background may work at any laboratory at the NIH. As mentioned previously, there is also a specialized clinical pharmacology track within this program.

This year proved to be a very difficult year for training programs and for the NIGMS staff who administer them. The budget rescission request for the Department of Health and Human Services submitted by the new Administration in March of this year included the elimination of trainee-related expenses, trainee travel costs and indirect costs. Because this budget revision came midway in the fiscal year, in order to meet the financial constraints imposed by the rescission budget, approximately half of the training programs in the Pharmacological Sciences Program suffered reductions in the committed number of trainee positions. While some funds for restoration did become available in the third quarter of the fiscal year, it was necessary to keep the ongoing training programs at the FY 1980 level of number of trainees. While every effort was made to keep the program directors apprised of these events as they occurred, the timing of the situation made planning and recruitment extremely difficult, especially in the area of clinical pharmacology.

ORGANIZATION AND MANAGEMENT

During FY 1981, the Pharmacological Sciences Program welcomed Dr. Warren C. Jones to the staff as a program administrator. Dr. Jones, who has postdoctoral and academic research experience in the area of peptide synthesis, will have responsibilities in both the biorelated chemistry and pharmacology areas. Ms. Patricia LeStrange joined the support staff during FY 1981. Miss Ellen Chuang, a summer student worker, helped to assemble this annual report. In addition to Dr. Jones, Drs. Carl A. Kuether and Christine K. Carrico serve as contact points for applicants and grantees of the Program. Dr. William Z. Potter, Coordinator for Clinical Pharmacology in the Pharmacology Research Associate Program, provided consultation and direction for PRAT fellows, both basic and clinical pharmacology fellows, and organized a well-received seminar series for all NIH scientists interested in pharmacological research.

RESEARCH HIGHLIGHTS

1. "The Role of Receptors in Glucocorticoid Action"
   GM 28825 (Feldman) Stanford University

   Several laboratories have recently noted the existence of vertebrate-like hormones in simple organisms including fungi and unicellular eukaryotes. However, the presence of hormone receptors analogous to those found in higher organisms has not been previously demonstrated. Dr. Feldman has investigated Candida albicans, a simple yeast.

   His results demonstrate the existence of a protein binder in the cytosol of C. albicans which has high affinity and considerable specificity for vertebrate steroids and which exhibits properties consistent with a receptor molecule. It is stereospecific, is extremely selective, and has a high affinity for [^{3}H]corticosterone which is of the same order of magnitude as mammalian glucocorticoid receptors. The number of binding sites (730 fmol/mg protein) is in the range of receptor binding capacity reported for some mammalian glucocorticoid systems.

Lipid extraction of C. albicans or its culture medium yielded a material which reversibly competes for yeast cytosol binding sites as well as mammalian receptor binding sites. These investigators hypothesize that this substance represents an endogenous ligand for the cytosol binder in the species.

These results suggest that C. albicans possesses both components of a hormone-receptor system, i.e., a ligand and a binding protein. The evolution of the mammalian steroid hormone-receptor system may stem from an analogous system in simple unicellular eukaryotic yeast.

The ability of mammalian steroids to interact with the C. albicans binder, and of the yeast lipid extractable factor to compete for mammalian binding sites, suggests the possibility of important symbiotic interactions between infecting organism and host. C. albicans infects humans and has a special predilection for patients receiving glucocorticoid therapy. It is possible that the hormonal milieu of the host may act via the C. albicans receptor to alter the infecting yeast. Conversely, the endogenous ligand from C. albicans may interact with host steroid receptors.

2. "Developmental Organizers of Drug Metabolism"
GM 26222 (Shapiro) University of Pennsylvania

Treatment of adult animals with a variety of neuropharmacological agents, such as hypnotics and sedatives, is able to modify sexual behavior, pituitary gonadotropin secretion, and estrous and menstrual cycles, and to induce pseudopregnancy and lactation. The effects on sexual maturation following prenatal exposure to xenobiotics have received little attention in spite of this fact. In an effort to understand the biochemical mechanisms involved in neuroleptic teratogenicity, Dr. Shapiro and his collaborators have studied the effects of phenobarbital on the sexual development of the rat. Pregnant rats were injected with phenobarbital, and the reproductive development in the female pups was studied. Phenobarbital significantly delayed the onset of puberty as determined by the time of vaginal opening and the first estrus. The results of estrous cycle analysis showed a high percentage of animals in the experimental group with a persistent estrus (38%) or constant estrus (20%), compared with no abnormality in the control group. Prenatal treatment with phenobarbital also produced infertility in a large number of animals. In order to investigate the mechanism for reproductive dysfunction, circulating hormone levels in the blood of these animals were measured during the estrous phase of the adult cycle. Prenatal treatment with phenobarbital increased plasma concentrations of estradiol and progesterone and decreased luteinizing hormone (LH) when the rats were 3 months of age. Analysis of cytoplasmic estrogen receptor demonstrated a significant increase in receptor concentrations in the adult uterus.

Studies were also conducted to determine the sensitivity of male rats to the teratogenic effects of the barbiturate. Anogenital distance, an indicator of anatomic masculinization, was significantly shorter in phenobarbital treated pups than in controls, and testicular descent was delayed significantly. Fertility was significantly reduced in the male offspring that received phenobarbital in utero. In agreement with this, a significant

decrease in testosterone levels was also observed in the adult offspring. Moreover, the level of LH, the trophic hormone for testosterone, was also reduced in the animals that received phenobarbital in utero, compared with that in control males. The seminal vesicle showed a significant increase in cytoplasmic androgen receptor concentration.

It has been demonstrated that both acute and chronic administration of narcotics and hypnotics, including barbiturates, lower plasma levels of LH and testosterone in rodents and man. A similar decrease in the plasma concentration of testosterone and LH in the animals exposed to phenobarbital during the prenatal period was also observed. However, the effects on hormone production following drug administration in adult animals disappeared after the drugs were withdrawn, whereas the prenatal administration of phenobarbital produced a permanent effect.

3. "Molecular Basis of Cytochrome P-450 Induction"
GM 27151 (Waterman) University of Texas Health Science Center

The goal of Dr. Waterman's research is to investigate the molecular basis by which cytochrome P-450 concentration is increased following administration of inducing agents. To date, three different forms of cytochrome P-450 have been studied. Two of these forms of cytochrome P-450 reside in hepatic endoplasmic reticulum, cytochrome P-450b being induced by phenobarbital and cytochrome P-450c being induced by β-naphthoflavone. The third form of cytochrome P-450 resides in the inner mitochondrial membrane of the adrenal cortex. This form of cytochrome P-450 is designated cytochrome $P\text{-}450_{scc}$ as a result of its cholesterol side chain cleavage activity; it is found to be induced by ACTH. The picture which is emerging from these studies is that the same general mechanism of induction may be operative with different forms of cytochrome P-450 in different tissues although the specifics may be quite different for each case, depending on the chemical nature of the inducing agent.

The studies on induction of cytochrome P-450b and P-450c have been carried out using Sprague-Dawley rats. Following administration of a single dose of inducing agent (either phenobarbital or β-naphthoflavone) total liver RNA is isolated at different times and translated in a rabbit reticulocyte cell-free translation system. Newly synthesized cytochrome P-450b or cytochrome P-450c is immunoisolated from total translation products. In both cases, there is a lag period of approximately 12 hours before an increase in cytochrome P-450 synthesis occurs. The optimum synthesis of cytochrome P-450 occurs using RNA isolated 16 hours after administration of phenobarbital and represents a 3-fold induction of translatability of cytochrome P-450b mRNA. The results obtained on cytochrome P-450c induction by β-naphthoflavone are similar, with an optimum occurring at 16 hours and a 3-fold induction of translatability of cytochrome P-450c mRNA. In each case, the newly synthesized cytochrome P-450 is found to be identical in molecular weight and peptide pattern with the mature form purified from rat liver. At longer times following administration of inducing agent, a decrease in translatability of both cytochrome P-450b and P-450c mRNA is observed.

The synthesis of cytochrome $P\text{-}450_{scc}$ has been studied in cultured bovine adrenal cortical cells. Following attainment of confluent, monolayer cell

cultures, ACTH (10^{-6}M) is added to some dishes, others being maintained as controls. In this study RNA was isolated at different times following initiation of ACTH treatment, and total cell protein was radiolabeled at those same times. Using an antibody specific for cytochrome $P\text{-}450_{scc}$, the newly synthesized form was immunoisolated from total, cell-free translation products and from newly synthesized (radiolabeled) cellular proteins. During the first 12-hour time period there is no change in translatability of cytochrome $P\text{-}450_{scc}$ in the cell culture, a result similar to that observed in rats for cytochromes P-450b and P-450c. However, in the case of cytochrome $P\text{-}450_{scc}$, the optimum synthesis (6-9 fold induction) is observed at 36 hours and then decreases at longer times. Furthermore, newly synthesized cytochrome $P\text{-}450_{scc}$ immunoisolated from the cell-free translation system is found to be a precursor protein with a molecular weight 5,500 daltons larger than the mature form, not an unexpected result for mitochondrial proteins.

In summary, while differences are observed in the timing of the inductive events between different forms of cytochrome P-450, a general pattern is emerging for the induction of cytochrome P-450. Following administration of the inducing agent, a lag period is observed prior to increased cytochrome P-450 synthesis. The observation that increased translatability of mRNA is observed for induction of cytochrome P-450b, P-450c and $P\text{-}450_{scc}$, leads to the conclusion that phenobarbital-, β-naphthoflavone-, and ACTH-mediated transcriptional synthesis of mRNA occurs during this lag period. The mechanisms by which these three different chemicals lead to increased transcriptional synthesis are probably quite different; however, increased transcriptional synthesis may be associated with induction of all forms of cytochrome P-450.

4. "Molecular Basis of Receptor-Cyclase Coupling"
GM 27800 (Bourne) University of California at San Francisco

Dr. Henry Bourne and his co-workers have been studying a membrane protein -- termed the N protein -- that is required for coupling hormone receptors to adenylate cyclase, the enzyme that synthesizes the second messenger, cyclic AMP. The protein is functionally absent from cells of a mouse mutant tissue culture line isolated in this laboratory. These investigators have now discovered that this mouse mutant cell line is a close counterpart to certain cells isolated from victims of pseudohypoparathyroidism (PHP), an inherited human disease.

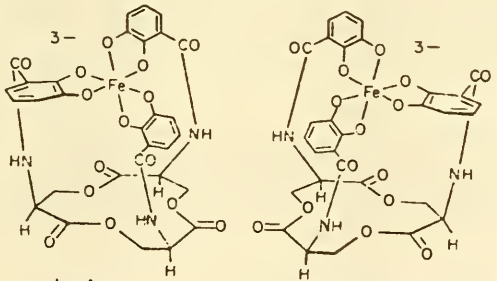
PHP patients show markedly decreased metabolic responses to hormones, such as parathyroid hormone, thyroid stimulating hormone, antidiuretic hormone, and glucagon, that work via cyclic AMP as a second messenger. Dr. Bourne's laboratory measured N protein activity in extracts of PHP cells, using a complementation procedure in which cell extracts are used to repair the N deficiency in membranes of the mouse mutant cell. In studies of 45 patients, three classes of cells from PHP patients -- erythrocytes, platelets, and cultured skin fibroblasts -- showed approximately 50 percent less N activity than did corresponding cells from normal controls. Surveys of kindreds with PHP showed that the biochemical defect correlated perfectly with expression of the disease.

In these studies PHP in several additional families was found to be associated with normal N activity in erythrocytes and fibroblasts. Recent studies, conducted in collaboration with Dr. Paul Insel at the University of California, San Diego, indicate that intact platelets from these patients accumulate much less cyclic AMP in response to hormones than do platelets from normal individuals. Thus this second group of PHP patients suffers from a biochemical lesion which, while not arising from defect in N protein activity, nonetheless impairs their ability to convert hormonal signals into the ubiquitous second messenger, cyclic AMP.

5. "Syntheses of Enterobactin and Enantioenterobactin"
GM 28828 (Rastetter) Massachusetts Institute of Technology

Microbes have evolved specialized ligands or "siderophores" for the acquisition and transport of insoluble Fe(III) into the cell. Enterobactin, the cyclic trimer of N-(2,3-dihydroxybenzoyl)-L-serine, is a siderophore which is overproduced by Escherichia coli and related enteric bacteria under low iron stress. The catechol-based siderophore forms a ferric complex, 1, which is bound and transported into the bacterial cell by a membrane receptor protein.

The total synthesis of enterobactin has been achieved by Dr. Rastetter's group, starting from L-serine. If D-serine is used, the enantiomer of the naturally occurring iron chelator is obtained (D-seryl enterobactin or enantioenterobactin). Measurement of circular dichroism spectra indicates the Δ-cis configuration for the Fe(III) enterobactin complex 1. As expected, enantioenterobactin forms the Λ-cis complex 2.



1, Δ-cis Fe(III)-enterobactin

2, Λ-cis Fe(III)-enantioenterobactin

The synthetic availability of the unnatural complex 2 has allowed these investigators to determine the stereospecificity of the E. coli membrane receptor protein.

Dr. Rastetter has examined both synthetic enterobactin and enantioenterobactin for their ability to transport iron in E. coli. Natural enterobactin and the material synthesized from L-serine are essentially equivalent in supporting the growth of E. coli RW193, a mutant deficient in the synthesis

of the natural siderophore. In contrast, enantioenterobactin (D-seryl enterobactin) inhibits the growth of strain RW193, apparently by binding iron in a form not accessible to the natural transport system. Competition experiments indicate that not only transport but also binding of Fe(III)• D-seryl siderophore enterobactin to the membrane receptor is significantly reduced as compared with the natural L-seryl siderophore complex having the Δ-cis configuration of the Fe(III)•catecholate center.

Apparently subtle changes in the geometry of Fe(III)•catecholate complexes can dramatically alter the complexes' biological activity. Knowledge of the specificity of bacterial membrane receptors can be used to advantage in rational drug design. For example, specific bacterial cells might be targeted through an antibiotic tailored to bind to a bacterial membrane receptor protein. Conversely, the uptake of chelated iron might be prevented with ligands not recognized by bacterial membrane receptors. Such ligands could find use in the treatment of Cooley's anemia, a human blood disease.

6. "A New Dopamine-Like Drug for Treatment of Congestive Heart Failure"
GM 22220 (Goldberg) University of Chicago

In previous studies in dogs in Dr. Goldberg's laboratory, propyl-N-butyl dopamine (PBDA) was found to differ from dopamine in that it does not stimulate the heart by a beta-adrenergic mechanism; it exhibits weaker alpha-adrenergic (vasoconstrictor) activity and produces greater reduction in sympathetic nervous system activity. In the kidney, however, PBDA retains dopamine-like vasodilating activity. These unusual properties suggested that the drug should be beneficial for the treatment of certain cardiovascular conditions. Accordingly, animal data were submitted to the FDA and the drug was approved for initial human studies. Dr. William Fennell and his associates at Baylor School of Medicine, in collaboration with Dr. Goldberg, administered PBDA to six patients with congestive heart failure in concentrations of 10 and 20 μg/kg/min. Beneficial effects were observed consistent with clinical improvement. These included increase in the output of the heart and decrease in both the elevated left ventricular pressures and peripheral resistance. On the basis of these studies, additional investigations are now being conducted. PBDA represents a totally different approach to the treatment of congestive heart failure and possibly hypertension as well.

7. "Deacetylation of N-Acetylprocainamide in Man"
GM 22371 (Atkinson) Northwestern University

N-Acetylprocainamide (NAPA) is a metabolite of procainamide (PA) that has been shown by several investigators to suppress cardiac arrhythmias in patients. NAPA may actually be preferable for long-term antiarrhythmic management of these individuals because it is less likely to cause elevated antinuclear antibody titers and the systemic lupus erythematosus-like reaction that is the major drawback to chronic PA therapy. Presumably, acetylation of the primary amine group of the PA molecule blocks metabolic formation of a toxic intermediate that is responsible for PA-induced lupus. If this is so, the safety of NAPA therapy should depend on the extent to which NAPA is deacetylated to PA.

Studies in rhesus monkeys indicate that as much as 20% of an administered NAPA dose is deacetylated to PA. Dr. Atkinson and his associates administered NAPA labeled with ^{13}C in the acetyl group to an investigator volunteer in order to distinguish between the ^{13}C-NAPA that was unmetabolized and NAPA that was formed by re-acetylation of PA. ^{13}C-NAPA and NAPA concentrations were determined by quantitative gas chromatography/mass spectrometry. Urine concentrations of PA, NAPA and the N-dealkylated metabolite of NAPA, NAPADE, were measured by high-pressure liquid chromatography. Pharmacokinetic analysis indicated that only 2.8% of the ^{13}C-NAPA dose was deacetylated to PA. The rate of deacetylation in this individual was also consistent with the low PA plasma concentrations that were measured in 4 patients actually receiving long-term NAPA therapy.

These results are considerably different from those reported in rhesus monkeys. However, the demonstration that NAPA is partially deacetylated to PA suggests that the apparent immunological safety of NAPA is relative rather than absolute. Most probably, a PA plasma concentration threshold exists and positive antinuclear antibody titers and lupus-like reactions will occur if this threshold is exceeded.

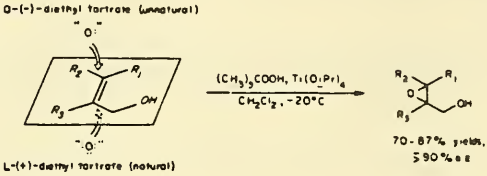
Investigators at the clinical pharmacology research center supported by NIGMS at Vanderbilt University were the first to identify desethyl N-acetylprocainamide (NAPADE) in the urine of a patient treated with procainamide (PA). Drs. Ruo and Thenot in Dr. Atkinson's laboratory developed a high-pressure liquid chromatographic method to measure NAPADE concentrations in the plasma of patients treated with PA and with N-acetylprocainamide (NAPA). Kinetic analysis of these measurements led to the conclusion that NAPADE is formed more rapidly from PA than from NAPA and to the prediction that a hitherto unidentified PA metabolite, desethyl procainamide (PADE), is an intermediate in the formation of NAPADE from PA. This prediction was subsequently confirmed by using GC-MS to identify PADE in the urine of a patient treated with PA.

Using a model of chloroform-induced ventricular fibrillation in mice to compare the relative antiarrhythmic potency of PA and NAPA with that of the de-ethylated metabolites PADE and NAPADE, PA was found to be 1.5 times more potent than PADE with respect to dose and 1.7 times more potent than PADE with respect to plasma concentration. NAPA was 1.8 times more potent than NAPADE with respect to dose and 2.9 times more potent with respect to plasma concentration. Measurement of PA, PADE, NAPA and NAPADE concentrations in the plasma of 10 patients receiving long-term PA therapy suggests that only PA and NAPA made important contributions to the antiarrhythmic responses that were observed.

8. "Asymmetric Epoxidation"
GM 28384 (Sharpless) Stanford University

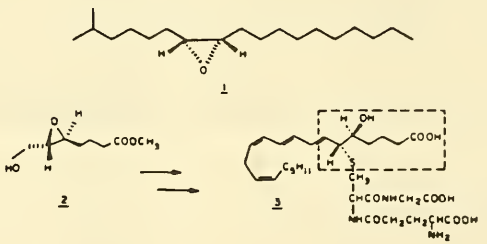
Enantioselective synthetic methods have been long sought after with only occasional partial success. Recently, Dr. Sharpless and his coworkers have discovered a new metal-catalyzed asymmetric epoxidation process which is highly stereoselective.

Most olefins are prochiral, and the possibility of selectively attaching an oxygen atom to one of the two enantiofaces of the olefin has long appealed to synthetic chemists. The reason for this is that the epoxide functional group is one of the most useful intermediates in organic synthesis because there are regio- and stereoselective methods both for constructing it and for controlling its subsequent reactions. The one great challenge which was yet to be met was the formation of enantiomerically pure epoxides from achiral olefins. The general and practical solution to this problem for the class of olefins which bear an allylic hydroxyl group (discovered by Dr. Sharpless and his coworkers) is as follows:



This new chiral epoxidation system possesses a number of unusual features. 1) It gives uniformly high asymmetric inductions throughout a wide range of substitution patterns in the allylic alcohol substrate. 2) Upon use of a given tartrate enantiomer, the system delivers the epoxide oxygen from the same enantioface of the olefin regardless of the substitution pattern on the olefin. 3) The simplicity of this new method is another of its attractive aspects. The necessary components [(+) or (-)-diethyl tartrate, titanium tetraisopropoxide, and tertiary butyl hydroperoxide] are all commercially available at low to moderate cost.

As a demonstration of the utility of this process, a number of natural products have been synthesized, including the Gypsy Moth sex attractant, (+)-disparlure (1), and also the epoxy alcohol (2) which is a key intermediate for synthesis of leucotriene C-1 (3), a slow-reacting substance of anaphylaxis.



The first commercial product to be made using this process is already on the market. J. T. Baker Chemical Company has licensed the Stanford methodology to synthesize the sex attractant of the Gypsy Moth, (+)-disparlure,

which is being used in traps now sold in garden supply stores. The U. S. Department of Agriculture has an extensive monitoring system for the Gypsy Moth, and each year this requires several hundred grams of (+)-disparlure. The former syntheses were difficult, so the price had reached about $1500 per gram. This new synthesis should reduce the price to about $50 per gram, saving the government several hundred thousand dollars a year. Because the discovery was made with NIGMS support, the government does not pay royalties for the use of the process.

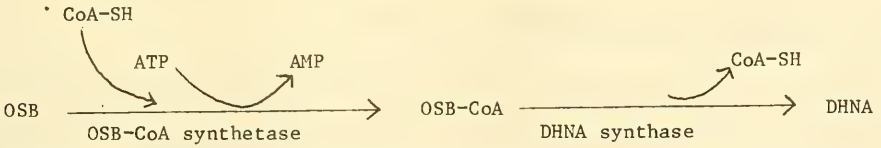
There will certainly be other applications of this process in the chiral insect pheromone field, but by far the majority of significant uses will be in syntheses of chiral drugs and vitamins.

9. "The Biosynthesis of Vitamin K"
GM 20053 (Bentley) University of Pittsburgh

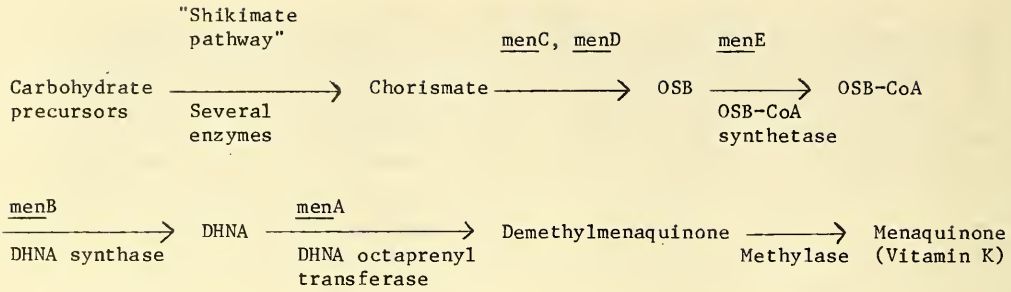
The fat soluble vitamin, vitamin K, is an indispensable component of the blood clotting process. The sources of the vitamin are bacteria normally present in the intestine, and plant materials. Dr. Bentley's objective has been to understand the mechanisms by which the vitamin is biosynthesized in bacterial systems. It is known to be derived from the important compound, chorismic acid, and two intermediates, o-succinylbenzoic acid (OSB) and 1,4-dihydroxy-2-naphthoic acid (DHNA), are required. The general outlines of the biosynthetic pathway are as follows:

Chorismate + α-ketoglutarate ⟶ OSB ⟶ DHNA ⟶ Vitamin K

In earlier work, this group of investigators has demonstrated that the conversion of OSB to DHNA requires the presence of ATP and CoA, and that two separate enzymes are involved:



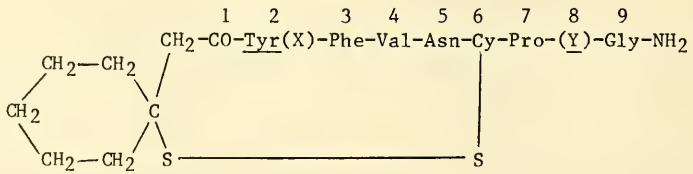
Other workers have shown the existence of four classes of menaquinone (= vitamin K, abbreviated as men) mutants in bacteria. In collaboration with Dr. H. Taber of Albany Medical College, it has been shown that certain mutants of Bacillus subtilis lack the OSB-CoA synthetase enzyme, and others the DHNA synthase enzyme. The gene responsible for the synthesis of OSB-CoA synthetase is termed menE, and for DHNA synthase, menB. This work has been extended to the more classical case of Escherichia coli mutants in collaboration with Drs. Guest and Shaw of the University of Sheffield, England. In this work, evidence for the menE and menB genes controlling synthesis of the two enzymes was also obtained. Genetic studies in this case have established that the menB and menE genes form part of a cluster of four genes controlling the early stages of vitamin K biosynthesis. The genetic enzymological evidence indicates the following picture for vitamin K biosynthesis in various bacteria.



Despite the evidence for the sequence shikimate → chorismate → OSB → OSB-CoA → DHNA → MK, there has never been a direct study of the biosynthesis of OSB itself. OSB is not accumulated to any significant extent in the culture medium of wild type strains of bacteria. Dr. Bentley and his co-workers have now used the menB mutant of E. coli, AN209, which accumulates OSB, to examine directly the incorporation of carbon atoms from glutamate (presumably converted to α-ketoglutarate) into this interesting benzenoid derivative. Samples of labeled glutamic acids were added to growth media, and the o-succinylbenzoic acid was isolated and converted to a dimethyl derivative which was purified on thin layer chromatograms and by gas chromatography. When the glutamic acid used as precursor contained ^{14}C at position 5, or was uniformly labeled, the dimethyl o-succinylbenzoate contained radioactivity (as shown by radiogas chromatography). However, from 1-^{14}C glutamate, the dimethyl o-succinylbenzoate was without radioactivity. Hence, in the biosynthesis of o-succinylbenzoate, carbon atom 1 of glutamate is lost, and carbon atoms 2, 3, 4, and 5 are retained.

10. "Design and Synthesis of Neurohypophyseal Peptides"
GM 25280 (Manning) Medical College of Ohio

Dr. Manning, in collaboration with Professor Wilbur H. Sawyer, has developed the first known antagonists of in vivo antidiuretic response to exogenous and endogenous arginine vasopressin (AVP). Such antagonists have been sought in these and other laboratories for over 28 years. These antagonists, which were designed by modifying a previously described highly potent and selective antidiuretic agonist [1-deamino, 4-valine, 8-D-arginine] vasopressin (dVDAVP) at positions 1, 2 and 8, have the following general structures.



Analogs 1-4: X=Me, Et, iPr, nPr; Y=D-Arg
Analogs 5-8: X=Me, Et, iPr, nPr; Y=L-Arg

All eight analogs cause a transient antidiuresis when injected i.v. into rats and effectively antagonize antidiuretic responses to subsequent injections of AVP. Antagonist activities are expressed as pA_2 values. The pA_2 represents the negative logarithm to the base 10 of the average molar concentration of an antagonist which reduces the specific biological response (in this case the antidiuretic response) of a given dose of agonist by 50 percent. These eight analogs exhibit the following antidiuretic pA_2 values: (1) 6.68; (2) 7.10; (3) 6.88; (4) 6.60; (5) 7.35; (6) 7.57; (7) 7.32; (8) 7.29. Thus analog (6) is the most potent of these antagonists, which are also potent antagonists of the vasopressor responses to AVP. These antagonists are potentially useful pharmacological tools for studies on the roles of AVP in regulating water balance in normal and pathophysiological states in animals and humans. They may also have clinical value for the treatment of hyponatremia secondary to the inappropriate secretion of AVP. Furthermore, they are excellent lead compounds for the design of even more potent and selective antidiuretic antagonists.

PHYSIOLOGY AND BIOMEDICAL ENGINEERING PROGRAM

SCOPE and OBJECTIVES

The Physiology and Biomedical Engineering (PBME) Program encompasses research on the application of the engineering, physical, chemical, biochemical, and physiological sciences to the solution of significant biomedical problems, as well as the development of new instruments and methods to facilitate such research. The Program ranges from laboratory research on cells and tissues to clinical research, and includes certain aspects of behavioral science.

The Program objectives are threefold. New knowledge and understanding of biologic function at the organ and organismic levels is sought, in order to better enable the physician to prevent, diagnose, and treat a variety of clinical conditions, including those following trauma or burn injury. The second objective is to develop new instruments and methods applicable to research in any of the Institute's programs. Finally, the Program supports the research training of a sufficient number of potential investigators in order to meet the Program's research objectives.

Although the staff of the Program has remained stable, the organization was modified by merging two of the three sections of the Program into the Biomedical Engineering and Instrument Development Section, led by Dr. Sue Badman. The professional staff of this section consists of Drs. Al-Aish, Cassman, Rivera and Shapiro. The other section, Physiological Sciences, remains intact, with the following staff: Drs. Black, O'Hern, Taylor and Van Lenten, under an Acting Chief, Dr. Leo von Euler.

In order to keep up-to-date, staff members have attended scientific meetings in their respective disciplines, and several have significant involvement in relevant national scientific societies.

Overall, the program budget for FY 1981 was about $46 million, roughly the same as in FY 1980. The figure for FY 1981 does include the P41 awards (about $1.1 million) that resulted from the instrumentation initiative of 1979. Although administratively placed in PBME, these awards are designed to advance research in all the programs of the Institute. The initiative was originally announced as a once-only competition, but because of the continuing need for such expensive, sophisticated instruments, and the laudatory comments from both Congress and the scientific community, the Institute staff has now set up a continuing program for such special instrumentation awards.

The Program continues to emphasize studies of general biologic significance, with the goal of uncovering physiologic or pathologic mechanisms. Acknowledging the essential role of astute clinical observation and careful description, the objective is to understand the underlying biologic processes so that more purposeful treatment and, ultimately, prevention of disease becomes possible. Severe trauma results in a bewildering series of systemic reactions in the human body, many of them excessive and harmful; and causes and effects must be unraveled for truly effective treatment to be designed.

The biologic emphasis is present in the research and development of instrumentation and methodology, as well, but, in addition, the Program lays stress on

projects that develop new concepts rather than on refinements or validation of existing technology.

RESEARCH

1. Biomedical Engineering and Instrument Development Section

The goals of this section include the application of engineering knowledge to the solution of significant physiological and other biomedical problems, as well as the development of new instruments and devices for physiological, basic and clinical research. In addition to supporting innovations in instrumentation, this section is concerned with the development of supportive methodologies in the areas of cellular and molecular biology, biochemistry, biophysics, and radiologic imaging of biologic structures.

Examples of current research supported by the section include physiological systems analysis, biomechanics, and physiological instrumentation. Other examples are the development of chemical and physical separation techniques, spectroscopic techniques and their extension to the study of ultrafast molecular phenomena, and microscopy involving all types of radiation.

Some Recent Trends

Progress in instrument development is often slow in that it involves developments in a number of different disciplines as well as communication among them. An example of this phenomenon occurs in reviewing recent trends in the field of bio-effects of ultrasound. While there have been no definitive demonstrations that diagnostic levels of ultrasound have deleterious effects upon their human subjects, current research does continue to demonstrate some abnormal processes of biological significance at power levels and time durations of exposure within the range of current use for diagnosis. It is clear that these processes cannot be attributed solely to thermal effects, but may be in part caused by the less well understood phenomenon of cavitation and the presence of microbubbles. The specter of adverse biological effects which may arise after longer term usage of ultrasound acts as an impetus for those involved in instrument development to strive for imaging devices which produce better resolution in shorter time intervals at lower power.

Recent advances in mass spectrometry may soon make possible the mass spectrometric analysis of relatively involatile compounds with masses in the 1500-20,000 dalton range. This should make more amenable the analysis of synthetic and natural polymers such as RNA's, DNA's, and small peptides. Concomitant advances in the volatilization of materials of interest under sufficiently gentle conditions must be linked with high performance instruments capable of high sensitivity at the higher mass ranges.

Several different laboratories are now making efforts to use NMR to image living material, either *in vitro* or *in vivo*, in order to follow metabolic or physiologic processes in living cells or tissues, whole animals, and eventually humans.

Shared Instrumentation

The NIGMS Program for support of shared instrumentation was announced in January 1979, with a March 1, 1979, deadline for submissions. Requests were limited to analytical instruments in a cost range of $40,000-$150,000. A total of 212

applications were received for review. These were considered by seven Special Study Sections, each considering a different category of equipment. Seventy-nine applications were funded, for a total of $9 million. These awards were made to 60 academic institutions in 29 states and the District of Columbia. Two-thirds of the awards were in the areas of NMR and electron microscopy, with the remainder distributed among other areas of spectroscopy, protein sequence analysis, mass spectroscopy, x-ray analysis, and cell sorting.

The current awards are now, in most cases, entering their third year. Delays in obtaining the instruments and in making them fully operational have made their routine use a recent event. The utility of this program will be evaluated during FY 1982.

The program was recently re-initiated, and 133 applications will be reviewed at the October 1981 meeting of the National Advisory General Medical Sciences Council.

2. Physiological Sciences Section

Trauma and Burns

The primary objective of the trauma/burns subprogram is to support research on the mechanisms of the body's systemic response to injury. The efforts to solve these problems incorporate physiologic, biochemical, and immunologic techniques, and the investigators supported are drawn from many disciplines. The attempts to understand the basic processes are hampered by the usual restraints of clinical research, which are especially severe when one is dealing with trauma victims, for "scheduling" is almost impossible, and the progression of the patient's condition often is very rapid. For these reasons it is necessary that the investigator have an active trauma service available for his studies, and many of the projects supported are conducted in conjunction with the relatively few large trauma treatment centers of this country. It is, of course, also important that the research be conducted in a setting that can provide up-to-date intellectual and technical resources of the highest quality. Parallel to the clinical research, the subprogram supports research using suitable animal models as well as tissue and cell culture material, where the drawbacks of limited applicability to the human condition are balanced by the ability to control a greater number of significant variables. The overall complexity is such that progress has been slow and generally at the empirical level. Even so, impressive improvements in reducing morbidity and mortality from trauma and burns have been made during the past 15 years.

With regard to burns, progress in research and treatment was reviewed in 1978 at a consensus meeting sponsored by NIGMS. The confereees also made recommendations which were described in the Institute's Annual Report of 1979. In November 1980, the Institute sponsored a follow-up meeting of the conferees which is described on page 10 of this report, as well as in a supplement to the August 1981 issue of the Journal of Trauma. The results of these conferences have been widely circulated and are thought to have had a significant impact on the clinical use of specific burn treatment approaches. In order to confirm this impression, an impact study of the 1978 Conference on Burn Care was conducted this year. It revealed that the level of ongoing research in the areas of need identified by the conferees was relatively low. A small number of the 25 burn treatment centers queried in this study, all of which are members of the 6 demonstration sites of the National Burn Demonstration Project, did report plans to begin research in one or more of the five identified areas.

A variety of grant mechanisms are used by this component of the program. In order to encourage recently trained investigators to undertake trauma/burn research, two mechanisms are available. The R23 New Investigator Research Award facilitates the transition from research training to independent scientific investigation. The K04 Research Career Development Award allows young investigators to concentrate on research at a crucial, initial stage of their careers by providing salary support for a five-year period to individuals who will bridge the gap between the clinical and basic sciences. In addition to these, the usual mechanisms of individual research project, program project, and research center grants are available. Currently two program projects and nine research center grants are being funded.

Anesthesiology

The main objective of the anesthesiology subprogram is to support research on the pharmacology of agents relevant to the practice of anesthesiology, with particular stress on the basic mechanisms of action of anesthetics and the systemic effects of anesthesia. The disciplinary methods and concepts of biochemistry, pharmacology, and physiology are used to approach research problems such as the effect of anesthetics on homeostatic mechanisms, chronic and acute toxicity of anesthetics, and the nature of the interaction of anesthetics with cell membranes. As in the area of trauma/burns, the full range of support mechanisms is used in anesthesiology, including R23 and K04 awards.

Behavioral Sciences and Adaptation

The objective of this subprogram is to support research on certain fundamental aspects of behavioral science, including studies that relate to the clinical research on trauma and burns, and anesthesiology. The most basic area supported is concerned with the biology of pain, and this research was given a major boost during the past decade with the discoveries of opiate receptors and the role of endorphins. With this stimulus, renewed efforts have been made to chart and to assess the respective roles of the many components of the nervous system involved in pain perception. Analogously, the biological substratum of behavior, generally, is being analyzed, using relatively simple animal models at this stage. Morphological and biochemical tools are being supplemented significantly by genetic approaches. Studies of adaptation focus on the behavioral effects of major environmental factors, such as heat, cold, and light; but adaptation in this sense is just a subset of behavior, generally, and the studies have broad implications.

Two types of award mechanisms, only, are used: research project and program project grants.

RESEARCH TRAINING

Predoctoral: The training program in Systems and Integrative Biology (SIB) plays an important role in the NIGMS training picture. As part of the PBME program it has provided predoctoral training in both physiology and biomedical engineering. Programs are multidisciplinary but may emphasize one or the other discipline. There has been a strong insistence that biomedical engineering programs provide a strong biological orientation so that trainees may truly enter the world of biomedical research. There has been a similar emphasis on appropriately quantitative training for the more physiologically oriented trainees.

The SIB training also has a place in the overall institute training approach. The SIB programs concentrate on biomedical problems above the cellular level, in many respects taking up where the Cell and Molecular Biology training grants leave off. There is often a meshing of the neurobiological interests of the many SIB preceptors with the interests of preceptors in the Pharmacological Sciences training program. Since several of the SIB programs have a neurobiological emphasis, appropriate for a program stressing integration, regulation and interactions, the trainees may continue in research areas not supported by NIGMS. This is consistent with the NIGMS intent to prepare basic researchers for all areas, including those relevant to the categorical institutes.

NIGMS has long been a supporter of bioengineering research and training. The institute has recognized the unique contributions which engineers can make to biomedical science. Review of several bioengineering training programs has exposed several special problems, some unexpected. It is the nature of the specialty that trainees have large course loads, since in the better programs they are expected to be both capable engineers and knowledgeable biological scientists. The reviews have also underlined the difference in approach of the two disciplines. In a sense, engineers are trained to solve problems and biomedical scientists to search for problems. One of the difficulties in existing training in the area is that the students rarely make the full transition, nor do they appear likely to in their future careers. They tend to be geared to solving problems, particularly clinical problems, in collaboration with clinical scientists, usually research physicians. A related limitation is that during graduate training trainees often tend to focus on a narrow clinical problem, one not necessarily central to biomedical science.

The NIGMS is trying hard to find the means of fostering the entry of the highest quality students into a career in biomedical engineering. Despite the difficulties cited there has been some marked success, and we do note that bioengineering is still a young field with many of the first generation of scientists still active. We feel that with critical review and program management the SIB program can support some of the best future scientists.

Postdoctoral: There has been little change in the level of support for postdoctoral research training through individual or institutional NRSA awards. A large proportion of the trainees or fellows in the trauma/burns or anesthesiology programs are physicians seeking at least two years of basic training in laboratory research. Several have received academic appointments following their period of training, and some are continuing their research on New Investigator Research Awards (NIRA).

RESEARCH HIGHLIGHTS

1. "CNS Sites of Action of Analgesic Drugs"
   GM 25998 (Levy) University of Illinois College of Medicine

   Cyclic nucleotides have previously been shown to cause potent analgesia following microinjection into the lateral ventricle of the rat brain. When administered in this manner, the nucleotide has access to the entire brain. This investigation has centered on a search for those local areas of the rat brain which contain neuronal substrates activated during this analgesic action and particularly on the question of whether these substrates are the same as those activated during analgesia produced by morphine.

The dibutyryl derivatives of the cyclic adenosine and guanosine 3':5' monophosphates (db cAMP and db cGMP respectively) have been shown to gain access to the intracellular space, where endogenous cyclic nucleotides exert their actions. db cAMP and db cGMP were microinjected locally in two areas of rat brain which previous studies had indicated contained active sites for analgesia produced by morphine: the reticular formation of the caudal brain stem (CRF) and the periaqueductal gray (PAG) of the midbrain. Dr. Levy also injected cyclic nucleotides into the intrathecal space of the lumbar spinal cord since morphine has been shown to act at the spinal level as well as at higher levels of the neuraxis. Nociceptive threshold was assessed, prior to and at intervals following microinjection of drugs, by the tail flick (TF), paw pinch (PIN), and hot plate (HP) tests.

Microinjection of 10 μg of db cAMP at sites in the CRF and PAG caused a pronounced elevation of nociceptive threshold as measured in all three tests. This analgesia had an immediate onset of action and reached a peak value within 10 minutes. Similar analgesia was also seen after injection of db cGMP at sites in the CRF, but not at the PAG sites, and neither db cAMP nor db cGMP caused analgesia when injected into the lumbar intrathecal space.

The correspondence between active sites for db cAMP and morphine suggests the possibility that endogenous cyclic nucleotides may somehow be involved in the mechanism of morphine's action, but the nature of this involvement is not clear. Cyclic nucleotides have been implicated as second messengers or modulators at monoaminergic and cholinergic synapses, and the mechanism of the analgesia produced by administration of these agents may reflect an action at such junctions located along pathways which control nociceptive threshold. Detailed knowledge of how this threshold can be modulated by manipulation of cyclic nucleotides would form a theoretical basis for the design of new analgesic agents.

2. "Lung Water and Pulmonary Capillary Permeability"
GM 27229 (Lewis) University of California, San Francisco

Studies of pulmonary capillary permeability were carried out in a canine model of sepsis, and the effects of elevated pulmonary capillary hydrostatic pressure and length of time after the septic insult were evaluated independently. Earlier demonstrations of permeability changes in this model had not allowed the effects of time to be separated from the effects of elevated pressures. Experiments have now demonstrated that both factors independently affect permeability, and have showed clearly that permeability changes are strongly time dependent. No evidence of permeability change is seen up to 8 hours after septic insult if pulmonary wedge pressure is kept below 15 mmHg. Between 15 and 20 mmHg, a permeability change appears to be developing at 8 hours but is not present at 6 hours. If wedge pressure is above 20 mmHg, the permeability lesion begins at 4 hours and continues to increase at 6 and 8 hours. The experiments were terminated at 8 hours with no evidence of a plateau in the permeability effects; it is assumed they would continue to increase. This is the first time that such temporal effects of sepsis have been clearly demonstrated, and this information should help considerably in reconciling earlier conflicting reports of the effects of sepsis wherein temporal effects were not evaluated or controlled for.

These experiments also point to the presence of an intermediary mechanism for the induction of permeability changes rather than a direct effect of bacteria or endotoxin. Agents which directly affect permeability have been independently evaluated (oleic acid, alloxan) and their effects are immediately apparent with a plateau in permeability changes at 3 to 4 hours. In contrast, sepsis does not cause any effects for 4 to 6 hours, requires a pressure in excess of 15 mmHg to become evident, and continues to increase at 8 hours.

Clinical studies have resulted in clarification of the causes of pulmonary edema after burns. In burned patients, hemodilution to colloid osmotic pressures below 10 mmHg does not cause pulmonary edema as long as hydrostatic pressures are not elevated. This is the first time that quantitative data regarding interstitial water content have been presented in clinical studies involving the question of colloid vs. crystalloid resuscitation. The results are definitive in showing that crystalloid resuscitation is not harmful after thermal injury. An additional conclusion allowed by this study is that there is no evidence for a burn "toxin" affecting the lungs, as has been proposed by some. Finally, the study clearly shows the predominant effect of sepsis in causing pulmonary edema after burns, with 3- to 4-fold increases in extravascular water content following the onset of sepsis.

3. "Mechanism of Action of Anesthetics"
GM 17536 (Feinstein) University of Connecticut

Dr. Maurice B. Feinstein noted some time ago that local anesthetics block many processes in cells and model systems that are calcium dependent, especially those that are activated by stimulation of cells. It is now recognized that many of these cellular activities involving calcium are not due to the cation itself but to its complex with calmodulin, an acidic protein found in all eucaryotic cells. The calmodulin-Ca^{2+} complex stimulates many enzymes involved in Ca^{2+} transport, contraction, glycogenolysis, lipolysis, protein phosphorylation and cyclic nucleotide metabolism. Feinstein's recent studies have shown that local anesthetics can abolish the effect of the calmodulin complex. Two of the systems studied were (a) Ca^{2+} ATPase-Ca^{2+} transport system of erythrocytes, and (b) brain cyclic nucleotide phosphodiesterase.

a) Ca^{2+} transport in erythrocytes (RBC) is carried out by an Mg^{2+}-dependent membrane ATPase that is stimulated by micromolar concentrations of Ca^{2+}. Ca^{2+}-ATPase activity was investigated as a function of both free Ca^{2+} concentration and calmodulin concentration. Local anesthetics were found to inhibit calmodulin stimulation but had little effect on basal ATPase activity.

   Inside-out RBC membrane vesicles can transport Ca^{2+} to their interior. On their exterior, the membrane surface now facing the medium, are found the enzyme sites for ATP and calmodulin. Thus free Ca^{2+} ATP and calmodulin concentrations can be varied and Ca^{2+} uptake can be measured. Local anesthetics were found to block calmodulin-stimulated Ca^{2+} uptake in these vesicles with little effect on basal Ca^{2+} uptake.

b) Cyclic nucleotide phosphodiesterase action on cyclic adenosine or guanosine monophosphate (cAMP or cGMP) is stimulated by calmodulin and the stimulation is antagonized by local anesthetics. The effects of the local anesthetics were found to be competitive vs. calmodulin but non-competitive vs. the substrate (cAMP or cGMP).

Although the diverse pharmacological effects of local anesthetics are presumed to be due to more than one mechanism of action, these studies have shown that some cellular actions of local anesthetics directed against Ca^{2+}-dependent processes may be due to a specific molecular action, the antagonism of calmodulin-dependent enzyme stimulation.

4. "High Resolution Autoradiography"
GM 10422 (Salpeter) Cornell University

The study of the intracellular synthesis and transport of secretory products was one of the earliest applications of electron microscope (EM) autoradiography to cell biology. By combining cell fractionation and EM autoradiographic procedures, the route of intracellular transport from the endoplasmic reticulum (ER) -> Golgi -> secretory granules has been defined most carefully in the guinea pig pancreas by Palade and his co-workers, and subsequently also in numerous other systems. The previous autoradiographic studies have left several questions unresolved. Among them are: 1) the extent to which the radioactive profiles ascribed to various organelles are affected by radiation spread from adjacent structures; 2) whether a cytoplasmic label, if present, can be distinguished from that in cell organelles; and 3) what is the true rate of movement of radioactivity from the various organelles. It has been reported by other workers to be very slow, especially in the rough ER, and the suggestion has been made that there may in fact be some nonexportable protein remaining in the ER.

These unresolved questions stem from technical difficulties in analyzing EM autoradiograms. Particularly bothersome is the fact that the percentage of developed grains falling outside a labeled organelle is inversely related to the size of that organelle. Furthermore, since all cell organelles are embedded in the cytoplasmic matrix, mutual radiation spread between the cytoplasmic matrix and the organelles is inevitable. The procedures for analyzing EM autoradiograms have been improving over the past ten years. Dr. Salpeter has been a major developer of these improvements, which stem from the utilization of high resolution emulsions and computer analyses to predict the grain distributions expected from variously labeled structures. By comparing these expected grain distributions with the observed grain distributions in an autoradiogram, the true grain sources can be identified, and their specific activities evaluated.

In the present study, Drs. Salpeter and Farquhar used the analysis technique devised by Dr. Salpeter and her co-workers to analyze autoradiograms of dissociated prolactin-secreting cells of the rat pituitary after pulse labeling with ^{3}H-leucine. After the cells were pulsed *in vitro* they were processed for EM autoradiography at times ranging from 30 minutes to 4 hours later. Using the fine-grained emulsion they were able to achieve a 1000 Å resolution. Their new method of analyzing autoradiograms is now capable of resolving the lumina of the ER and Golgi from the ground substance. Their results demonstrate the same secretory pathway (rough ER -> Golgi -> immature granules -> intermediate granules -> mature granules) as previously described by Dr. Farquhar using conventional emulsion and analysis. However, the precision with which the label could be localized within an individual organelle was much greater than achieved previously, and the time resolution was much sharper.

Their new results are summarized below:

1. The radioactivity dropped essentially to zero in the ER by 30 minutes, in the Golgi by 1 hour, and in the immature granules by 2 hours postpulse. This is considerably faster than suggested by previous reports. There was no significant residual radioactive material in any of these compartments.

2. Residual nonexported material was found to be located in the cytoplasm, not in the ER as previously reported.

3. The peak total radioactivity was essentially the same in the immature granules as later appeared in the intermediate granules. This suggests that there is a total transfer of material from the immature granules to the intermediate granules with no loss from the immature granules to any other compartment and no input into the intermediate granules from any source other than immature granules. This fits the currently accepted model for intracellular transport and processing of secretory proteins.

4. The conversion of immature granules to intermediate granules is faster than the conversion of intermediate granules to mature granules. This represents a longer time period spent as intermediate granules during which, presumably, final processing of the proteins is taking place.

Still unresolved are questions pertaining to intracellular traffic, such as the pathways taken by secretory proteins and membrane proteins to and through the Golgi complex and pathways taken by recycling membranes from the cell surface to other cell compartments. It is envisioned that this EM autoradiographic approach will be useful, in conjunction with other studies, to resolve these questions and allow investigators to determine the influence of various factors which control secretion and to pinpoint with greater accuracy the sites of their effect.

5. "Circular Dichroism and Circular Intensity Differential Scattering from the Nucleus of an Intact Cell"
GM 26857 (Salzman) University of California

The state of condensation of chromatin in a mammalian cell nucleus changes as the cell passes through its life cycle. These changes are correlated to cell growth, differentiation, and function. Dr. Salzman and his colleagues at the Los Alamos Scientific Laboratory have investigated chromatin condensation states in the nuclei of single Chinese hamster ovary cells (CHO) using a special microscope adapted to a commercial circular dichrograph.

Some molecules such as DNA differentially absorb and scatter light of opposite circular polarizations. The absorption phenomenon is called circular dichroism (CD), and the scattering phenomenon is referrred to as circular intensity differential scattering (CIDS). A microscope with strain-free optics has been adapted to a Cary 60 circular dichrograph at the Lawrence Berkeley Laboratory. This instrument measures the summed CIDS and CD signals integrated over a large solid angle in the forward direction as a function of wavelength from 200 to 340 nm. This microscope has been used to measure the CD/CIDS spectrum from single nuclei in intact cells. The cells were selected in each of the major phases of the cell cycle.

The CD/CIDS signals were large and reproducible. Near 270 nm the signal increased monotonically as a function of increasing optical density. The rate of increase was rapid across G1 and slower across S and G2. The scattering may be accounted for as that due to the supercoiled helices present in condensed chromatin.

CD/CIDS spectra were also obtained for the nuclei of single superficial squamous epithelial cells. The spectra of these highly condensed pycnotic nuclei were characterized by very strong signals, which were quite distinct from those of any of the CHO cell nuclei.

Dr. Salzman and his group are now developing a flow cytometer cell sorter with which circular intensity differential scattering measurements will be made on individual mammalian cells over a limited angular range. This tool may be useful for investigating changes in chromatin conformation under the stress of low doses of environmental mutagens.

6. "Development of Tandem Mass Spectrometry"
GM 28254 (Enke) Michigan State University, and
GM 16609 (McLafferty) Cornell University

Mass spectrometry is one of the most powerful tools available for analysis of organic compounds. To be effective, however, the samples to be analyzed must first be separated into reasonably pure components. The most common approach has been to link a gas chromatograph to the spectrometer. Coupling of a liquid chromatograph has been, and is still being, attempted, with limited success. Another approach is the use of the mass spectrometer itself to perform the separation. This is known as tandem, or MS/MS, spectrometry. There are numerous advantages of this approach, compared with coupling to a chromatograph. Larger and more polar molecules can be analyzed, and, most importantly, MS/MS is much faster the GC/MS.

McLafferty and his colleagues at Cornell were among the first to develop this technique. They used a "reverse-geometry" double-focusing MS, placing the magnetic sector before the electrostatic sector. The mixture is "soft ionized," usually by chemical ionization, producing primarily the molecular ion. These are separated by their mass/charge ratio in the magnetic analyzer. The ion selected is then fragmented by collisional means, and the kinetic energy of the fragments measured by the electrostatic analyzer to produce a mass spectrum. Djerassi (GM 06840, GM 20276, GM 28352) has used this form of MS/MS to separate and identify steroid mixtures. Most recently, McLafferty has constructed a high-resolution MS/MS, where each stage is double-focusing, (e.g., an MS/MS//MS/MS).

This approach, although valuable, is very expensive. A more reasonable cost, together with increased resolution over conventional MS, was achieved by Enke and his collaborators. They have developed a triple-quadrupole MS, (e.g., MS/MS/MS). Quadrupole spectrometers are simple mass filters, cheap and easy to use. Enke's system has a soft ionization system (chemical ionization/ electron impact) which feeds a parent ion into the first mass filter. The selected ion is then fragmented by collision with a neutral molecule in the strong focusing field of a quadrupole. The fragment ions are then analyzed in a third quadrupole acting as a mass filter. This MS/MS/MS system is easy to use, very sensitive with good resolution, and cheap.

Potential applications of MS/MS have been growing rapidly in recent years, and commercial operations are now, or will soon be, producing a variety of instruments based on this approach.

7. "Composition of Weight Gain in Surgical Patients"
GM 26396 (Elwyn) Columbia University

The interrelationships between the intake of bulk dietary constituents and growth or maintenance of body tissues has concerned both nutritional biochemistry and medicine for decades. The understanding of these interrelationships has led to the definition of minimum requirements for protein and energy intake that serves as the foundation of current nutritional practice. A renewed interest in nutrition has been stimulated by the development of practical methods for the administration of long-term intravenous nutrient regimens, particularly as they apply to adult surgical patients who have temporarily lost either their ability to take food by mouth or their ability to digest and absorb the food taken in. Despite the progress that has been made in the use of total parenteral nutrition (TPN) over the past decade, there is still insufficient information to define the optimal amounts of carbohydrate, protein and fat suitable for the various clinical conditions requiring intravenous feeding.

Dr. David H. Elwyn and co-workers, as part of their efforts to develop more quantitative guidelines for the use of nutritional therapy, have recently examined some of the problems, particularly respiratory complications, that may result from the overfeeding of one or more of the major nutrients. The old adage, "more of something good is not necessarily better," appears to apply. Although both dietary carbohydrate (glucose) and protein (amino acids) are needed to restore the metabolic health of an individual, overfeeding of either of these foodstuffs can result in frank respiratory failure and, in the extreme, cardiopulmonary collapse and death.

Diet interacts with respiration in a variety of ways. Differing diets affect the amount of CO_2 produced and the patient's ventilatory response to this change in CO_2 production. These investigators have found that carbohydrate is the major dietary constituent which affects CO_2 production. This fact is related to the body's inability to store large amounts of carbohydrate as glycogen, and therefore, except for transient changes in these glycogen stores, essentially all dietary carbohydrate must be oxidized to CO_2 to meet immediate energy needs or converted to fat for later usage as an energy source. In the absence of any dietary intake, at steady state, the energy needs of the body are met essentially by the oxidation of fat. This is a metabolic process with a respiratory quotient (RQ) of 0.7 (volume of CO_2 produced/volume of O_2 consumed). As dietary carbohydrate is introduced and increased until there is no net fat oxidation and carbohydrate becomes the main energy source, CO_2 production increases steadily and the RQ increases to 1.0. Further increases in carbohydrate intake result in the conversion of glucose to fat, a process with an RQ of 8.7. Overall, there is an increase in CO_2 production as the energy needs of the body are increasingly met by the oxidation of carbohydrate and dietary carbohydrate is stored as fat. In going from a fasting state to a glucose-based TPN regimen providing 3,000 calories of glucose per day, CO_2 production doubles solely because of the change in the fuel used by the body to meet its energy needs. This increase in CO_2 production with increasing calorie intake can be avoided to the extent that dietary fat can replace carbohydrate as an energy course.

The fact that dietary amino acids play a major role in increasing respiratory drive presents another difficult nutritional problem since there is no substitute for protein or amino acids in the restoration of lean body mass. One of the more important factors leading to pulmonary insufficiency in patients is the deterioration and weakness of the respiratory muscles resulting from nutritional depletion. It is a common practice to provide large quantities of foodstuffs to such individuals with the hope of rapidly restoring their respiratory muscles and thereby their respiratory function. This practice can be self defeating since the resulting increased CO_2 load and ventilatory drive may result in pulmonary failure. It therefore may not always be possible to restore depleted muscle at a rapid rate. For the patient with little pulmonary reserve, the investigators think it appropriate to limit energy intake to the level of energy expenditure and possibly to provide the calories in part as fat rather than totally as carbohydrate. The amount of amino acids administered should depend on how well they are tolerated. As long as respiratory distress or the need for mechanical ventilation can be avoided, amino acid intake should be maintained at an appropriate level for optimal restoration of lean body mass. With any signs of respiratory distress, intake should be reduced. Although the overall aim of a feeding program is to restore the patient's health as rapidly as possible to that which existed before the injury occurred, overfeeding introduces its own complications. A more restricted aim, simply preventing further loss of muscle tissue, may be more appropriate than rapid restoration.

CONTRACTS

The support of contracts and interagency agreements constitutes a relatively minor activity of the PBME Program.

1. "Reconstruction and Testing of a Superconducting Electron Microscope"
(Contract N01 GM 2116)
Duke University ($270,000, FY 1981 Funds)

A contract was awarded to Duke University on June 30, 1980, with Dr. J. David Robertson as Principal Investigator and Robert Worsham, the original builder of the microscope, as Project Engineer.

Phase I involves reassembly and restoration of the microscope. When the microscope is reassembled the investigators will demonstrate to a site visit team its operation at the contract specifications--its previous level of performance (14 months; $249,067 total cost).

Upon such a satisfactory demonstration the contract will proceed to Phase II, evaluation of the microscope. A new Project Engineer is being sought for the remainder of the contract. Phase II requires the investigators to demonstrate in a systematic way: 1) the improvement in electron exposure that can be tolerated by glucose embedded catalase, or a similar specimen, at liquid helium temperature compared with that obtained at liquid nitrogen and room temperature; 2) that high resolution electron diffraction patterns can be obtained from crystals of biological interest using the superconducting microscope; and 3) whether the instrumental contrast transfer function extends to better than 3 Å. With these results a complete evaluation of the practical potential of the superconducting microscope for contributing new information on high resolution biological structure will be obtained as well as an

evaluation of its performance as a potential "user-oriented" instrument (1 year; $336,327 total cost).

Since, if Phases I and II are wholly successful, the future use of the microscope is expected to be supported through a research or research resource grant, Phase III provides support for one year, keeping the microscope intact and under vacuum to allow an orderly transition to such support.

The scientific evidence for the need for this instrument has been strengthened by recent preliminary results reported from Europe. These data show preservation of high resolution at 4° K of 50-100 times that at room temperature, and confirm some previous reports from a different European laboratory. There is, at present and for the near future, no other electron microscope in this country with a high resolution, super-conducting stage capable of performing biological studies.

2. "Refurbishment and Renovation of the Infrared Heterodyne Ratiometer"
(Contract N01 GM 52124)
Andros Incorporated (funded with FY 1980 Funds; no 1981 Funds)

The generally accepted method for assaying the stable isotope of carbon (atomic weight of 13 instead of 12) is by high resolution mass spectroscopy, requiring instrumentation costing in excess of $250,000. NIGMS funds were used to construct a $^{13}CO_2/^{12}CO_2$ Ratio Analyzer using innovative ideas from NASA - Ames and the Andros Corporation in the hope of developing an alternative analytical instrument and procedure as sensitive and accurate as the mass spectrometer but less costly to manufacture, maintain and operate. This ratiometer is being prepared for use in the laboratory of Dr. Charles Irving, Baylor College of Medicine, who responded successfully to a request for applications issued by the program. This laboratory will use this ratiometer to gather important human data on the production of $^{13}CO_2$ after ingestion of ^{13}C labeled compounds and will validate the new analytical procedure.

MINORITY ACCESS TO RESEARCH CAREERS

SCOPE

The Minority Access to Research Careers (MARC) Program is institutionally based and is designed to assist minority colleges and universities in the training of greater numbers of scientists and teachers for careers in the biomedical sciences. It was designated as a formal program of the National Institute of General Medical Sciences in December 1975. Applications assigned to this program are reviewed by the Minority Access to Research Careers Review Committee, which was chartered specifically for the review of such applications, and which is composed of consultants including minority representatives from appropriate scientific fields, chosen primarily from the academic community.

OBJECTIVES

The primary objective of the program is to increase the number of minority scientists engaged in biomedical research while helping to strengthen the research and teaching capabilities of these minority institutions. The support mechanisms used to accomplish this mission are: the MARC Faculty Fellowship, the MARC Visiting Scientist Award, the MARC Honors Undergraduate Research Training Program, and the MARC Predoctoral Fellowship.

PROGRAM COMPONENTS

The MARC Faculty Fellowship program--the first of the four MARC support mechanisms -- provides opportunities for advanced research training for selected faculty members of four-year colleges, universities and health professional schools in which student enrollments are drawn substantially from ethnic minority groups. These institutions may nominate faculty members to apply for MARC Fellowships for support for a period of up to three years of advanced study and research training in graduate departments and laboratories, either as candidates for the Ph.D. degree or as investigators obtaining postdoctoral research training in the biomedical sciences. Faculty Fellows are expected to return to the nominating institutions at the completion of such training.

MARC Faculty Fellows are selected on a competitive basis. Evaluation is based upon the applicants' qualifications and potential for research and teaching, as evidenced by academic records, reference reports and publications, as well as by a research training proposal, the proposed training situation (i.e., the sponsor's and the institution's training facilities and staff), and other relevant information.

The second of the four support mechanisms, the MARC Visiting Scientist Award, has as its purpose the provision of financial support that allows outstanding scientist-teachers to serve as visiting scientists at four-year colleges, universities, and health professional schools where student enrollments are drawn substantially from minority groups. The primary intent is to strengthen research and teaching programs in the biomedical sciences for the benefit of students and faculty in these institutions by drawing upon the special talents of scientists from other institutions. Reciprocal benefits should accrue to

the MARC awardee through the added experience gained by involvement in innovative science education and research development programs.

An applicant's proposal must include arrangements for the visiting scientist to reside in the campus community and to participate fully in programs of teaching, development of research, and/or counseling as outlined by the institution. Evidence of negotiations and of some tentative agreement between the applicant institution and the scientist teacher nominated to serve as the MARC Visiting Scientist should be provided in the application. Individuals nominated as Visiting Scientists in this program should be recognized biomedical scientist scholars. Proposals may request support for the MARC Visiting Scientist Awards for periods ranging from an academic quarter to a maximum of one year. Stipends are determined on an individual basis, according to the nominee's current salary or other possible source of stipend support for the proposed period in residence.

The third mechanism of support is the MARC Honors Undergraduate Research Training Grant Program. This activity was initiated at the suggestion of Congress and Institute consultants and staff, as a means of emphasizing the value and importance of providing biomedical training at the undergraduate level in minority institutions. The objectives of the program are to increase the number of well-prepared students who can compete successfully for entry into graduate programs leading to the Ph.D. degree in the biomedical sciences, to help develop a strong science curriculum, and to stimulate an interest in undergraduate research as preparation for graduate study in the biomedical sciences.

Training support is offered to highly selected undergraduate honors students at four-year colleges, universities, and health professional schools in which student enrollments are drawn substantially from ethnic minority groups (such as American Indians, Blacks, Hawaiians, Mexican Americans, and Puerto Ricans). Each institutional grant will have a maximum support period of five years. These honors programs for third and fourth year students should be designed to improve significantly the research training capabilities of the minority institutions. Applications for support should contain mechanisms to augment and improve the science curricula, strengthen the faculty, and improve laboratory facilities. Funds are available for research equipment and supplies essential to the program, and for stipends, tuition, fees, and limited travel costs for the trainees. Arrangements for special training at universities and laboratories other than the grantee institution are encouraged and should be described in the application.

The newest component of the Program, the MARC Predoctoral Fellowship Program, provides support for research training leading to the Ph.D. degree in the biomedical sciences for selected students who are graduates of the MARC Honors Undergraduate Research Training Program. It is expected that such training will be conducted in graduate-degree programs of the highest quality. Support is not available for individuals enrolled in medical or other professional schools, unless they are enrolled in a combined degree (M.D.-Ph.D.) program. Awards are conditional upon acceptance into a specified doctoral (Ph.D.) degree program in biomedical research.

MARC Predoctoral Fellows are selected on a highly competitive basis. A maximum of up to five years of support may be recommended, based on the merit of the application and evidence of satisfactory progress in the doctoral program in which a successful applicant is enrolled. The award provides an annual stipend to the student and funds to help defray the expense of tuition, fees and supplies.

HIGHLIGHTS

Since the beginning of the MARC Faculty Fellowship Program in 1972, 151 individuals have received support under the program. In that nine-year period approximately 360 applications for MARC Faculty Fellowship support were received, and 317 were reviewed for scientific merit. Of the 79 predoctoral and 72 postdoctoral award recipients, 112 have completed their traineeship period. Of the 79 predoctoral fellows, 35 (approximately 45%) have now received the terminal degree, and 99 (88%) of those having completed the program (112) have returned either to the original home institution or to another minority institution. Of the 72 postdoctoral fellows 62 have completed training and 56 (90%) have returned to minority institutions.

The average length of time spent in predoctoral training was 2.9 years. Postdoctoral training has averaged 1.3 years.

The institutions that served as training sites have included some 70 universities, research laboratories, and federal institutions in 32 states and 5 foreign countries. The home institutions are broadly representative, including 68 universities in 23 states and the Commonwealth of Puerto Rico. Upon returning to their home institutions, an increase in rank was obtained by 52 fellows. Of the 52 fellows continuing in their previous rank, 20 were initially at the full professor level. Of the 32 fellows starting at the instructor's level, 26 (81%) have risen in rank, 16 (50%) having risen one rank and 10 (31%) having risen two ranks to associate professor. Fellowships were awarded to 29 assistant professors, of whom 14 (48%) became associate professors and 2 became full professors. Of the 23 fellows who were associate professors when they received the award, 13 (56%) remained at the initial rank and 10 (44%) became full professors.

Since returning to their home institutions, four Faculty Fellows have been awarded research grants or have applications pending for a MARC Honors Undergraduate Program training grant. Significant numbers of Fellows serve as faculty advisors on several MARC and Minority Biomedical Support (MBS) Programs for undergraduate students.

The Visiting Scientist Award Program has been underutilized. Only seven awards have been provided, based on fourteen applications reviewed. Considerable advance planning by the host institution and the prospective visiting scientist is necessary. Such planning has probably tempered a fuller usage of this award. However, strong continuing efforts to advertise and encourage applications for the Visiting Scientist Award are being made by Program staff. The MARC Review Committee and the National Advisory General Medical Sciences Council strongly support continuation of this award mechanism.

To date, 168 applications for the Honors Undergraduate Research Training Program have been received, of which 86 have been approved, and 36 have been funded at minority institutions around the country. In 1980, there were 73 graduates, 66% of whom have applied to graduate school and 25% to professional school. In 1981, the program had 109 graduates, significant numbers of whom have applied to graduate schools.

This year, the newest component of the MARC Program, the MARC Predoctoral Fellowship, was initiated. It had been noted by Program staff that a number of students graduating from the Honors Undergraduate Program were reluctant to continue their education outside of a minority college setting, and that they were not as aggressive as other students in seeking out training opportunities.

In an effort to resolve this problem, NIGMS invited applications for Individual National Research Service Award MARC Predoctoral Fellowships. To date 16 applications have been received, of which 12 have been approved. It is expected that greater numbers of applications will be generated as the pool of MARC graduates enlarges.

EVALUATION

Assessment of improvements and accomplishments of the components of the MARC Program is a continuing effort on the part of Program staff. The scientific community, the MARC Review Committee, the National Advisory General Medical Sciences Council and special consultants cooperate in this effort through continued interaction with Program staff and other NIGMS staff.

Significant accomplishments of the Program are the following:

- The "honors" concept of the MARC Program has encouraged institutions that are recipients of awards to establish a different and more rigorous program for students in biology, chemistry, and in some cases mathematics. Many have incorporated an honors thesis requirement for graduating MARC students.

- The provision of funds for seminars and lectures, release time for faculty, and up-to-date equipment and sufficient supplies, as well as the addition of full-time and part-time faculty in disciplines not formerly represented, have all contributed to academic enrichment. In addition, specialized courses have been set up to prepare students to take standardized tests, an area where minority students have traditionally been weak.

- The scientific activities of grantee departments have been strengthened, and as a result, it has become more common for MARC faculty and trainees to be invited to present papers at local, regional, and national scientific meetings.

- The "public relations" aspects of the MARC Program, with their emphasis on research, have produced a new awareness of the possibilities available in biomedical research among students historically motivated towards careers in medicine, dentistry, and education.

- The fact that MARC students are performing well in graduate schools, thereby providing increasing numbers of "role models" for minority students, has resulted in a 50% increase in the number of science majors at those

minority institutions.

SUPPLEMENTARY PROGRAM ACTIVITIES

During the past year the MARC Program as part of its objectives has supported a number of conferences, all of which were designed to bring specific minority concerns into focus and thereby propose and initiate mechanisms by which these concerns may be addressed.

- In November 1980, the MARC Program, in conjunction with nine cooperating NIH Institutes, sponsored a conference entitled "Biomedical Research and Its Impact on the Hispanic and Indian Communities," organized by the Society for Advancement of Chicanos and Native Americans in Science (SACNAS) in Albuquerque, New Mexico.

  Research topics discussed included alcoholism, drug abuse, cancer, mental health, infectious diseases and ethnic medicine. Over 450 participants attended, among them a number of NIH scientists of Hispanic origin as well as students and scientists who are members of SACNAS, and additional representatives from the federal government and industry.

  Conference activities included technical symposia, selected demonstration projects by area high school students, a poster session by undergraduate and graduate student researchers, exhibits and tours of local scientific laboratories.

  Specific issues discussed by the conferees resulted in the following recommendations: continuing appraisal and attention to health problems in Hispanic and Indian communities, to determine the extent to which these problems may be tractable to biomedical research; ascertaining the biomedical research being conducted by minority group scientists and others interested in particular medical problems affecting Hispanics and Indians; promoting the growth of a network between Hispanic and Indian scientific communities and scientists in the mainstream biomedical research community; motivating and helping young Hispanics and Indians to pursue careers in science through role modeling and media publication; identifying financial support for students interested in pursuing science careers; providing advice on federal legislation to establish programs designed to train minority scientists; increasing participation of SACNAS members as directors, reviewers, and consultants of programs in science research and education; identifying key elements necessary to increase participation in biomedical research. Conference participants urged that future meetings would undertake a more focused approach, giving priority to any one of these concerns. They strongly encouraged the dialogue initiated among Society members and interested Federal agencies, academic and industrial representatives with a view to establishing closer working relationships.

- A meeting of the alumni of the Faculty Fellowship Awards was held at NIH in December 1980, under sponsorship of the MARC Program, to assess the success of the Faculty Fellowship program and chart a course for program improvements. A scientific seminar was included in the agenda, during which five distinguished intramural NIH investigators reported on their recent research activities. Additionally, two of the alumni presented scientific papers. The conference participants included 60 alumni, representatives

of the cooperating NIH Institutes and other interested NIH Staff.

During the meeting, the following facts emerged. Almost all of the Fellows had returned to their original home institutions. Most, but not all, institutions had honored their commitment to these fellows by keeping positions open for their return. Most fellows had received an increase in academic rank. A large number had taken on administrative duties in addition to starting research projects. Although some alumni had obtained research support from Federal agencies, except for the Minority Biomedical Support Program of the Division of Research Resources, they have received little NIH grant support to date. Many alumni are planning to submit research grant applications, or have pending applications at NIH and the National Science Foundation.

MARC Faculty Fellowship alumni recommended that the program should provide: support for short-term training periods, up to 3 months, for alumni; additional support after three or more years, for a sabbatical leave; regional meetings and workshops which would address research grant award procedures and requirements; some extension of time during the initial fellowship period to allow fellows to prepare an application for research grant support; transitional support to allow alumni to continue research efforts initiated during tenure of the fellowship upon arriving back at the home institution; help in establishing communication between neighboring majority institutions and faculty fellows when the latter return to their home institutions; and support for an annual conference to assess the accomplishments of the program.

- The MARC Program was responsible for coordinating the funding, by 12 Institutes and Divisions of NIH, for the "Conference of Puerto Rican Scientists in the United States" held in April 1981 under the auspices of the American Association for the Advancement of Science. Seventy-five Puerto Rican scientists, engineers, and students, mostly from the mainland U.S., presented background papers, held work sessions on topics arranged by educational level and served as panelists and speakers.

  Conferees noted the artificial distinction made between "mainland" and "island" and called for much closer interaction through formal and informal mechanisms, asking for a unified scientific association that includes both locations. In discussing educational issues, however, distinctions were emphasized between the two settings in matters of educational policy concerning the need for bilingual instruction, Spanish being the primary island language and English being the primary mainland language.

  Recommendations which were endorsed included the following: producing materials which publicize the achievements of Puerto Rican scientists to encourage Puerto Rican students to think about science careers; using local churches, Puerto Rican clubs, and Spanish language media to disseminate information about science and scientists; listing and publicizing good traveling exhibits which offer students "hands on" experience in science to motivate them to pursue further study in technical fields; updating and computerizing the directory of Puerto Rican scientists for use in role model projects, network creation, and peer review assignments by science agencies; starting career orientation programs which brief Puerto Rican (and other) students on graduate school application, financial

aid, and the availabilty of academic resources; increasing research experience opportunities for undergraduate students; and promoting student attendance at scientific meetings as a way to learn how scientists conduct their business and to establish contacts.

As a prelude to creating a national association, regional network centers were proposed for New York, California, the Rocky Mountain states, Boston, Philadelphia, and Washington, D.C., and possible future sites in Texas and the Chicago area.

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